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P27808.A04

**REMARKS**

Entry of the foregoing amendments is respectfully requested.

**Summary of Amendments**

Upon entry of the foregoing amendments, claims 21, 27, 31, 33 and 35 are amended. Claims 17-36 remain pending, with claims 17 and 26 being independent claims.

Applicants submit that entry of the present amendments is proper in that these amendments do not require a further search and do not raise any new issues, but rather reduce the number of issues.

Applicants emphasize that the amendments to claims 21, 27, 31, 33 and 35 are without prejudice or disclaimer, and Applicants expressly reserve the right to prosecute these claims in their unamended form in one or more continuation and/or divisional applications.

**Summary of Office Action**

As an initial matter, Applicants note with appreciation that the Examiner has withdrawn all objections and the claim rejections under 35 U.S.C. § 101, 35 U.S.C. § 112, second paragraph, and 35 U.S.C. § 102(b) set forth in the previous Office Action.

Claims 21, 27, 31, 33 and 35 are rejected under 35 U.S.C. § 112, first paragraph, because the specification, while being enabling for a method of treating allergic diseases,

allegedly does not reasonably provide enablement for the prevention of all allergic diseases.

Claims 17-36 are rejected under 35 U.S.C. § 103(a) as allegedly being anticipated by three newly cited documents, i.e., by Noguchi et al., U.S. Patent No. 4,933,323 (hereafter "NOGUCHI") in view of Tsuji et al., U.S. Patent No. 6,491,943 (hereafter "TSUJI") and further in view of the PDR document for omeprazole (hereafter "PDR").

**Response to Office Action**

Withdrawal of the rejections of record is respectfully requested, in view of the foregoing amendments and the following remarks.

***Response to Rejection of Claims under 35 U.S.C. § 112, First Paragraph***

Claims 21, 27, 31, 33 and 35 are rejected under 35 U.S.C. § 112, first paragraph, because the specification, while being enabling for a method of treating allergic diseases, allegedly does not reasonably provide enablement for the prevention of all allergic diseases. In this regard, the present Office Action essentially repeats the arguments set forth in the previous Office Action.

Applicants note that amended claims 21, 27, 31, 33 and 35 submitted herewith do not recite the prevention of allergic diseases, wherefore this rejection is moot.

Applicants emphasize that the amendments to claims 21, 27, 31, 33 and 35 are by no means to be construed as Applicants' admission that this rejection is of any merit. On

the contrary, Applicants still believe that for at least all of the reasons set forth in response to the previous Office Action, the present specification provides more than sufficient enablement for the prevention of allergic diseases by the claimed method. Accordingly, Applicants expressly reserve the right to prosecute claims drawn to the prevention of allergic diseases by the claimed method in one or more continuation and/or divisional applications.

***Response to Rejection of Claims under 35 U.S.C. § 103(a)***

Claims 17-36, i.e., all claims of record, are rejected under 35 U.S.C. § 103(a) as allegedly being anticipated by NOGUCHI in view of TSUJI and further in view of PDR. In this regard, the rejection essentially asserts that the abstract of NOGUCHI teaches a peptide possessing activity of inhibiting histamine release and IgE antibody production in the onset of type I allergies such as bronchial asthma, urticaria and allergic rhinitis, and that from TSUJI, col. 2, lines 14-16, it can allegedly be taken that certain green tea catechins suppress histamine release and can treat allergies. Apparently because present claims 20 and 26 (and the paragraph bridging pages 4 and 5 of the present application) identify catechin as a known proton pump inhibitor and because omeprazole also is a proton pump inhibitor, the Examiner takes the position that "the references combined show that proton pump inhibitors such as catechins, and likewise omeprazole, suppress or inhibit histamine release and that the inhibition of IgE dependent histamine release effectively treats allergic conditions such as asthma". Based on these assertions, the rejection alleges the subject

matter recited in the claims of record would have been obvious to one of ordinary skill in the art.

Applicants respectfully traverse this rejection. First, the present rejection amounts to the assertion that if one specific proton pump inhibitor (e.g., catechin) has been shown to inhibit histamine release and IgE antibody production, it would be obvious to one of ordinary skill in the art that, regardless of structure all (known and not yet known) proton pump inhibitors and specifically, the proton pump inhibitors recited in the present claims, are capable of inhibiting histamine release and IgE antibody production. The Examiner has not provided any documentary evidence whatsoever that would support such a far-reaching and general conclusion.

Also, if the above conclusion and the logic applied in the above rejection were correct, compounds like vitamins and anti-cancer agents which are known to have activities similar to those exhibited by catechin such as, e.g., reducing bacterial infection, anti-oxidant activity and anti-cancer activity, should all be assumed to inhibit histamine release and IgE antibody production and be useful for the treatment of allergies as well. That this is and can not be the case is apparent.

Further, Applicants note that, as already mentioned above, catechin is known for its various physiological activities including reducing bacterial infection, anti-oxidant and anti-cancer effect, etc. Also, catechin is reported to show anti-allergic activity by reducing the release of histamine secreted from basophiles or mast cells which play an important role in allergic reactions when they are stimulated by allergens. See, e.g., Biochemical and Biophysical Research Communications 274, pp. 603-608 (2000) and Biosci. Biotechnol.

Biochem., 62(7), pp. 1284-1289, 1998, copies of which are enclosed herewith for the Examiner's convenience.<sup>1</sup>

However, it is noted that, according to the reports up to now, the histamine release inhibiting effect of catechin is attributed to the fact that it inhibits the influx of calcium ions into cells or because it phosphorylates the tyrosine residue which plays an important role in the histamine release process. These two proposed mechanisms for the histamine release inhibiting effect of catechin have nothing to do with the proton pump inhibitory activity of catechin or any other compound recited in the present claims.

The present invention reveals for the first time the mechanism of the HRF secretion pathway and provides a novel method of inhibiting the secretion of IgE-dependent HRF for the treatment of allergic diseases caused by HRF. Accordingly, the disclosure of TSUJI that certain green tea catechins such as epigallocatechin gallate and epicatechin gallate suppress the liberation of histamine from mast cells clearly fails to teach or render obvious the present invention.

In other words, the reason why catechin is recited in present claims 20 and 26 is not because it inhibits the influx of calcium ions into cells or because it phosphorylates the tyrosine residue, but because it exhibits proton pump inhibitory activity (see, e.g., British Journal of Pharmacology (2000) 130, pp. 1115-1123 and abstract of J. Pharm Pharmacol., Nov. 1992, 44(11), pp. 926-8, copies whereof are also enclosed herewith<sup>1</sup>), i.e., in view of a

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<sup>1</sup> In accordance with M.P.E.P. § 609C(3), the documents cited above in support of Applicants' remarks are being submitted as evidence directed to an issue raised in the mentioned Official Action, and no additional fee or Certification pursuant to 37 C.F.R. §§ 1.97 and 1.98, or citation on a FORM PTO-1449 is believed to be necessary.

mechanism that is entirely different from the mechanisms proposed so far for the histamine release inhibiting activity of catechin.

Omeprazole is known as a selective proton pump inhibitor. The results reported in the present application provide evidence that proton pump inhibition can result in the prevention and treatment of an allergic effect associated with the secretion of IgE-dependent HRF. Specifically, the data in the present application provides evidence that pre-treatment with omeprazole significantly reduces the release of HRF, a protein involved in allergic reactions, and thus results in an anti-allergic effect.

To sum up, in view of the fact that to date the anti-allergic effect of catechin has been attributed to reasons different from its proton pump inhibitory activity and in view of the fact that it has never been shown before that certain proton pump inhibitors such as, e.g., omeprazole show an anti-allergic activity by inhibiting the secretion of HRF, none of the documents relied on in the present rejection renders obvious the subject matter of any of the claims submitted herewith.

There are even further reasons why the cited documents do not teach or suggest the claimed subject matter. For example, the peptide disclosed by NOGUCHI possesses a histamine release inhibiting activity. In comparison, the proton pump inhibitors of the present invention inhibit the secretion of IgE-dependent histamine releasing factor (HRF). In other words, the proton pump inhibitors employed according to the present invention have an indirect effect on histamine release, i.e., by inhibiting the secretion of HRF which is known to stimulate a basophil to induce histamine secretion (see, e.g., page 1, lines 10-12 of the present specification). In view of these

different mechanisms of inhibiting histamine release, there is no link between the peptide of NOGUCHI and the present proton pump inhibitors.

Further, both NOGUCHI and TSUJI fail to indicate the precise inhibition mechanism of histamine release associated with the substances mentioned therein. As one of ordinary skill in the art will understand, there are numerous conceivable mechanisms for the inhibition of histamine release. Accordingly, there is no motivation to combine the teachings of NOGUCHI and TSUJI for this reason alone.

It must also not be forgotten that a compound such as omeprazole, i.e., a sulfur-containing benzimidazole derivative, is structurally completely different from catechin (for example, catechin does not contain sulfur or any N-heterocycle). Accordingly, even if it had been known that the histamine release inhibiting activity of catechin is due to its proton pump inhibitory activity, in view of the significant structural differences between catechin and a compound such as omeprazole one of ordinary skill in the art would not have expected that omeprazole shares with catechin any of the other activities of catechin as well. Otherwise there would be no reason why omeprazole should not also exhibit all of the other activities of catechin such as, e.g., reducing bacterial infection, anti-oxidant and anti-cancer activity.

For at least all of the foregoing reasons, the rejection of present claims 17-36 under 35 U.S.C. § 103(a) over NOGUCHI in view of TSUJI and further in view of PDR is unwarranted, wherefore withdrawal thereof is respectfully requested.

## CONCLUSION

In view of the foregoing, it is believed that all of the claims in this application are in condition for allowance, which action is respectfully requested. If any issues yet remain which can be resolved by a telephone conference, the Examiner is respectfully invited to contact the undersigned at the telephone number below.

Respectfully Submitted,  
Chul-Hee LEE et al.



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## Epigallocatechin Gallate Inhibits Histamine Release from Rat Basophilic Leukemia (RBL-2H3) Cells: Role of Tyrosine Phosphorylation Pathway

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Some tea polyphenolic compounds including (−)-epigallocatechin gallate (EGCG) have been shown to inhibit histamine release from mast cells through poorly understood mechanisms. By using a mast cell model rat basophilic leukemia (RBL-2H3) cells we explored the mechanism of the inhibition. EGCG inhibited histamine release from RBL-2H3 cells in response to antigen or the calcium-ionophore A23187, while (−)-epicatechin (EC) had little effect. Increased tyrosine phosphorylation of several proteins including ~120 kDa proteins occurred in parallel with the secretion induced by either stimulation. EGCG also inhibited tyrosine phosphorylation of the ~120-kDa proteins induced by either stimulation, whereas EC did not. The tyrosine kinase-specific inhibitor piceatannol inhibited the secretion and tyrosine phosphorylation of these proteins induced by either stimulation also. Further analysis showed that the focal adhesion kinase pp125<sup>FAK</sup> was one of the ~120-kDa proteins. These findings suggest that EGCG prevents histamine release from mast cells mainly by inhibiting tyrosine phosphorylation of proteins including pp125<sup>FAK</sup>. © 2000 Academic Press

Mast cells and basophils play a central role in immediate allergic reactions mediated by immunoglobulin E (IgE). Binding of multivalent allergens to specific IgE bound to the high-affinity IgE receptor (Fc $\epsilon$ RI) on the mast cells or basophils leads to the release of inflammatory mediators such as histamine and serotonin, and arachidonic acid metabolites. These mediators ul-

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timately cause the various symptoms of allergy including dermatitis and asthma (1, 2).

Rat basophilic leukemia RBL-2H3 cells are mucosal mast cell type that is major model for the study of IgE-mediated degranulation (1, 3). These cells express Fc $\epsilon$ RI, a heterotetrameric receptor that consists of one IgE-binding  $\alpha$  subunit, one  $\beta$  subunit, and two disulfide-bonded  $\gamma$  subunits (2). Stimulation of RBL-2H3 cells with IgE and specific antigen can mimic cell activation by allergens under physiological conditions. Fc $\epsilon$ RI triggers a cascade of events that induce degranulation, lipid mediator release, cytokine secretion, contributing to allergic reactions (4).

Fc $\epsilon$ RI-mediated secretion involves complex biochemical reactions including the activation of protein tyrosine kinases, phospholipases, protein kinase C (PKC) isoforms, Ca $^{2+}$  influx, and cytoskeletal reorganization. Tyrosine phosphorylation of proteins is a critical event in the signaling pathways leading to secretion (5–7). These proteins can be divided into at least two groups based on their timing of tyrosine phosphorylation (8, 9). The tyrosine phosphorylation of one group of proteins precedes Ca $^{2+}$  influx and PKC activation, while that of the other group is downstream of these two events. The first group involves the  $\beta$  and  $\gamma$  subunits of Fc $\epsilon$ RI, Lyn, Syk, phospholipase Cy1,  $\gamma$ 2, and SLP-76. These proteins are important in the transduction of early signals following Fc $\epsilon$ RI aggregation. The second group includes the focal adhesion kinase pp125<sup>FAK</sup> (10) and paxillin.

Polyphenolic compounds such as catechins are ubiquitously found in green tea (11). The main polyphenolic compounds found in green tea are (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), and (−)-epigallocatechin gallate (EGCG). These polyphenolic compounds exhibit protective effects against bacterial infection, tumor promotion and

progression (12, 13). ECG, EGC, EGCG are recently shown to inhibit antigen- or the calcium-ionophore A23187-induced histamine release from RBL-2H3 cells, and among them EGCG is the most potent (14, 15). However, the mechanism of the inhibition is poorly understood. Since mast cells are activated to release chemical mediators, and tyrosine phosphorylation has been shown to play a pivotal role in the activation, we assessed the effect of EGCG on mast cell tyrosine phosphorylation. In this paper we show that EGCG inhibits tyrosine phosphorylation pathway, a critical event in signal transduction to secretion.

## MATERIALS AND METHODS

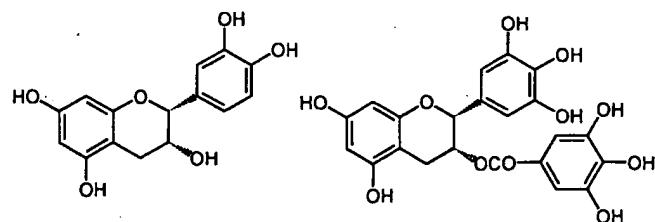
**Reagents.** Protein A-agarose beads, the calcium-ionophore A23187, horseradish peroxidase (HRP), and anti-dinitrophenyl (DNP) IgE monoclonal antibody (mAb) SPE-7 were obtained from Sigma (St. Louis, MO). EGCG and EC were from Kurita Industries (Tokyo, Japan). DNP-bovine albumin conjugate (33 molecules of 2,4-dinitrophenol coupled to 1 molecule of bovine serum albumin) and AG99 [tryphostin A46,  $\alpha$ -cyno-3,4-(dihydroxy)cinnamide] were obtained from Calbiochem (San Diego, CA). Piceatannol (*trans*-3,3',4,5'-tetrahydroxystillbene) was from BioMol (Plymouth Meeting, PA). The anti-phosphotyrosine mAb 4G10 was purchased from UBI (Lake Placid, NY). HRP-conjugated species-specific anti-mouse and anti-rabbit immunoglobulins (Ig) were from Amersham (Bucks, UK). The anti-pp125<sup>FAK</sup> mAb A-17 was from Santa Cruz (Santa Cruz, CA).

**Cell culture.** The rat basophilic leukemia (RBL-2H3) cells obtained from NIH (JCRB) (cell number JCRB0023) were grown in Dulbecco's modified Eagle's minimal essential medium (DMEM) (Sigma) supplemented with 10% fetal calf serum (FCS) (GIBCO) in 5% CO<sub>2</sub>.

**Cell stimulation.** RBL-2H3 cells were harvested by incubating them in Hanks' balanced salt solution (HBSS) containing 1 mM EDTA, 0.25% trypsin for 5 min at 37°C. RBL cells were suspended in complete DMEM at concentrations of  $5 \times 10^5$  cells/ml, and plated on a 24-well plate at the density of  $2 \times 10^5$  cells/well. Then, the cells were sensitized with 1  $\mu$ g/ml of anti-DNP IgE overnight at 37°C. IgE-sensitized cells were washed with PBS and suspended in DMEM containing 20 mM Hepes, pH 7.4 (Hepes-DMEM). The cells were stimulated with 1  $\mu$ g/ml of DNP-BSA in Hepes-DMEM. After incubating for 30 min at 37°C, the supernatants were collected for histamine release. For analysis of overall tyrosine phosphorylation, cells were lysed with a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and processed as described below. For analysis of tyrosine phosphorylation of pp125<sup>FAK</sup>, cells grown in a 100-mm culture dish was stimulated with the agents tested for 5 min at 37°C and processed as described below.

**Measurement of histamine release.** IgE-sensitized cells were activated with DNP-BSA as described above. Histamine content in supernatants was determined using a commercially available enzyme-linked immunosorbent assay kit (ICN Pharmaceuticals) according to the manufacturer's protocol. To quantify the total amount of histamine in the cells, they were treated with 0.05% Triton X-100, and the extracts were analyzed for histamine release as described above. The amount of histamine in unstimulated cells was considered to be as the spontaneous release.

**Immunoblotting analysis.** Cellular protein tyrosine phosphorylation was determined by immunoblotting analysis using the anti-phosphotyrosine mAb 4G10 as described previously (16). Briefly, after stimulation, the cells were lysed with SDS-PAGE sample buffer, and the samples were boiled for 3 min. Samples were sub-



(-)-Epicatechin (EC) (-)-Epigallocatechin gallate (EGCG)

FIG. 1. Structure of EGCG and EC.

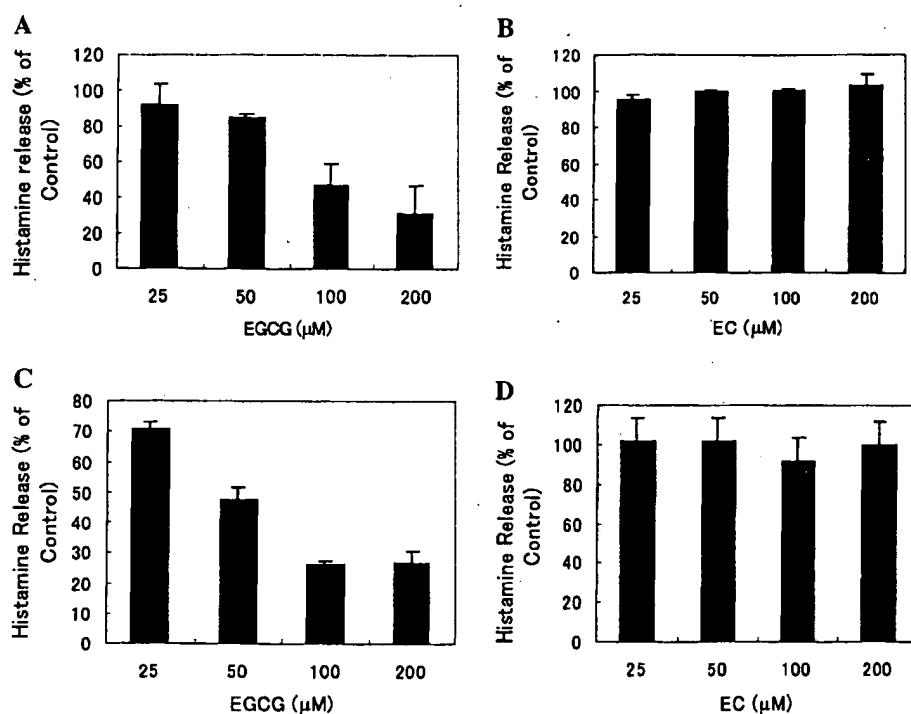
jected to SDS-PAGE using a 10% separation gel under reducing conditions and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). The PVDF membrane was incubated with 3% BSA overnight at 4°C. The PVDF membrane was incubated with 0.2  $\mu$ g/ml of the anti-phosphotyrosine mAb 4G10 for 1 h at room temperature and then with 0.1  $\mu$ g/ml of HRP-conjugated species-specific anti-mouse Ig (Amersham, Bucks, UK) for 1 h at room temperature. After extensive washing of the membrane, the immunoreactive proteins were visualized using the Enhanced Chemiluminescence kit (Amersham) according to the recommendations of the manufacturer. The PVDF membrane was exposed to Fuji RX film (Fuji Film, Tokyo, Japan) for 2 to 30 min.

**Immunoprecipitation.** When the tyrosine phosphorylation state of pp125<sup>FAK</sup> was analyzed,  $10^7$  cells were suspended in Hepes-DMEM and stimulated as described above. The cells were lysed with the ice-cold lysis buffer containing 20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 2 mM EDTA, 0.2 mM *p*-amidinophenylmethanesulfonyl fluoride, 20  $\mu$ M leupeptin, and 0.15 U/ml aprotinin. After centrifugation, the whole cell lysates were incubated with protein A-agarose beads overnight at 4°C. Cell lysates were incubated with 5  $\mu$ g of the anti-pp125<sup>FAK</sup> mAb A-17 for 1 h at 4°C and then incubated with protein A-agarose for 1 h. The beads were washed 3 times with lysis buffer and then resuspended in SDS-PAGE sample buffer and boiled for 3 min. The eluates from immunoprecipitates were subjected to immunoblotting analysis using the anti-phosphotyrosine mAb 4G10 or the anti-pp125<sup>FAK</sup>.

## RESULTS

### Effects of Tea Polyphenolic Compounds on Histamine Release from RBL-2H3 Cells

Preliminary experiments showed that specific antigen (DNP-BSA) induced histamine release from anti-DNP-IgE-sensitized RBL-2H3 cells in a concentration-dependent manner at all concentrations ranging from 0.25 to 4  $\mu$ g/ml. Almost the same extent of secretion was achieved with antigen at concentrations ranging from 1 to 4  $\mu$ g/ml. RBL-2H3 cells released  $17.0 \pm 3.1\%$  (mean  $\pm$  SD,  $n = 6$ ) of their total histamine content during a 30-min incubation with medium (the spontaneous release). RBL-2H3 cells released  $61.7 \pm 11.12\%$  (mean  $\pm$  SD,  $n = 6$ ) of their total histamine content in response to 1  $\mu$ g/ml antigen. The polyphenolic compounds, epicatechin (EC) and epigallocatechin gallate (EGCG) (Fig. 1), were tested for their ability to inhibit the secretion. Neither EGCG nor EC by itself affected the spontaneous release. However, EGCG inhibited antigen-induced histamine release in a concentration-



**FIG. 2.** Effects of EGCG and EC on histamine release induced by antigen or by A23187. RBL-2H3 cells plated in a 24-well plate were cultured overnight with or without 1  $\mu\text{g}/\text{ml}$  of anti-DNP IgE. IgE-sensitized cells were incubated with EGCG (A, C) or EC (B, D) at the concentrations indicated at 37°C for 30 min and then stimulated with 1  $\mu\text{g}/\text{ml}$  of DNP-BSA (A, B) for 30 min. IgE-untreated cells were incubated with EGCG (A, C) or EC (B, D) at the concentrations indicated for 30 min and then stimulated with 2  $\mu\text{M}$  A23187 (C, D) for 30 min. Histamine content in supernatants was determined by an ELISA method as described under Materials and Methods. Results are expressed as % of control that is treated with DNP-BSA or A23187 in the absence of polyphenolic compounds and are means  $\pm$  SD. The results presented are representative of four different experiments.

dependent manner. The histamine release was reduced by 61 to 89%, respectively in cells treated with 100 and 200  $\mu\text{M}$  EGCG (Fig. 2A). On the other hand, EC at all concentrations ranging from 25 to 200  $\mu\text{M}$  had little effect (Fig. 2B).

The calcium-ionophore A23187 induced histamine release from RBL-2H3 cells in a concentration-dependent manner at all concentrations ranging from 0.25 to 2  $\mu\text{M}$  with an optimal concentration of 1  $\mu\text{M}$ . RBL-2H3 cells released  $75.1 \pm 7.2\%$  (mean  $\pm$  SD,  $n = 6$ ) of their total histamine content during a 30-min incubation with 1  $\mu\text{M}$  A23187. EGCG also inhibited the histamine release in a concentration-dependent manner. A23187-induced histamine release was reduced by 57 and 84%, respectively, in cells treated with 50 and 100  $\mu\text{M}$  EGCG (Fig. 2C). By contrast, EC at all concentrations ranging from 25 to 200  $\mu\text{M}$  had little effect (Fig. 2D).

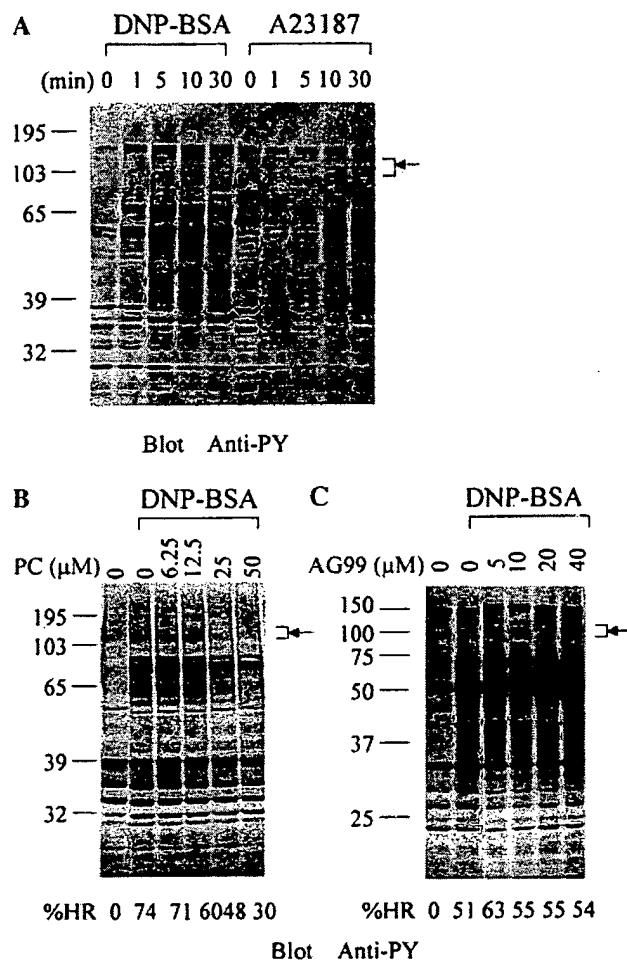
#### Role of Tyrosine Phosphorylation in Histamine Release from RBL-2H3 Cells

Immunoblotting of cell lysates with the anti-phosphotyrosine mAb 4G10 showed the occurrence of multiple tyrosine-phosphorylated proteins even in un-

stimulated cells. The extent of tyrosine phosphorylation was variable in different experiments. Despite this variability, a reproducible increase of tyrosine phosphorylation was observed following stimulation with antigen. In most experiments tyrosine-phosphorylated proteins with the apparent molecular masses of 55–60, 72,  $\sim$ 120, and 150 kDa were prominently observed (Fig. 3A). Time course experiments showed that the increases of the 55–60 and the 72-kDa proteins were relatively early events that could be observed within 1 min, after the stimulation, while the increase of the  $\sim$ 120 kDa proteins could be detected at 5 min.

On the other hand, a smaller number of tyrosine-phosphorylated proteins including the  $\sim$ 120-kDa proteins were detected after stimulation with A23187 (Fig. 3A). Also in this case the increase of the  $\sim$ 120-kDa proteins became clear at 5 min after the stimulation.

To validate the role of tyrosine phosphorylation in histamine release, we next examined the effect of tyrosine kinase-specific inhibitors on the secretion. The two different types of inhibitors, piceatannol and AG99 (tyrphostin A46) were tested for their ability to inhibit histamine release induced by antigen or by A23187. Piceatannol has been shown to inhibit the non-receptor



**FIG. 3.** (A) Tyrosine phosphorylation induced by antigen or by A23187. RBL-2H3 cells were sensitized with 1  $\mu$ g/ml of anti-DNP IgE as described in the legend to Fig. 2. The IgE-sensitized cells and IgE-untreated cells were incubated with 1  $\mu$ g/ml of DNP-BSA and 2  $\mu$ M A23187, respectively, at 37°C for the time indicated. After stimulation, the cells were lysed with SDS-PAGE sample buffer, and the samples were boiled for 3 min. The tyrosine-phosphorylated proteins from the cell lysates were analyzed by immunoblotting with the anti-phosphotyrosine (PY) mAb 4C10 as described under Materials and Methods. Briefly, samples were subjected to SDS-PAGE using a 10% separation gel under reducing conditions and transferred to PVDF membranes. The PVDF membrane was probed with the anti-PY mAb. After extensive washing of the membrane, the immunoreactive proteins were visualized using the ECL. (B, C) Effects of different tyrosine kinase inhibitors on histamine release and tyrosine phosphorylation induced by antigen. IgE-sensitized RBL-2H3 cells were incubated with piceatannol (PC) (B) or AG 99 (tyrphostin A46) (C) at the concentrations indicated at 37°C for 30 min and then stimulated with 1  $\mu$ g/ml of DNP-BSA. After stimulation at 37°C for 5 min (for analysis of tyrosine phosphorylation) or for 30 min (for histamine measurement), tyrosine phosphorylation and histamine content in supernatants were determined by immunoblotting using anti-PY and by an ELISA method, respectively. Percent histamine release (%HR) results are indicated at the bottom of each lane. Arrows indicate the ~120-kDa proteins. The results presented are representative of three different experiments.

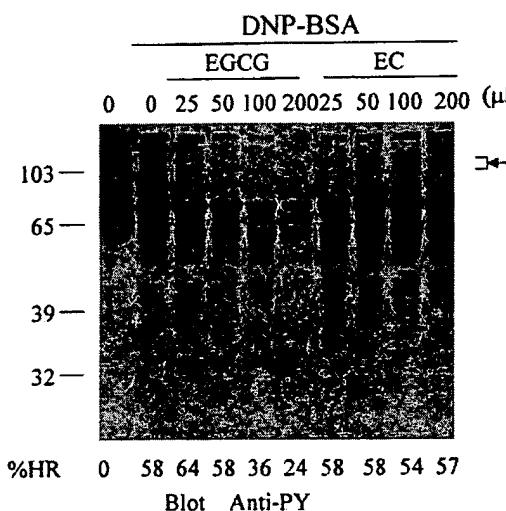
tyrosine kinase Syk preferentially (17). Cells were incubated with increasing concentrations of the compound for 30 min, and then stimulated with 1  $\mu$ g/ml

antigen or 2  $\mu$ M A23187. Piceatannol had little effect on the spontaneous histamine release at all concentrations ranging from 6.25  $\mu$ M to 50  $\mu$ M. However, the compound inhibited antigen-induced histamine release in a concentration-dependent manner (Fig. 3B). Furthermore, piceatannol also inhibited antigen-induced tyrosine phosphorylation in a concentration-dependent manner. Tyrosine phosphorylation of the ~120-kDa proteins was the most sensitive, and piceatannol inhibited histamine release and tyrosine phosphorylation of the ~120-kDa proteins with a comparable concentration (25  $\mu$ M). Piceatannol (50  $\mu$ M) inhibited antigen-induced tyrosine phosphorylation of the ~120-kDa proteins completely, whereas the compound inhibited the secretion strongly (more than 60%) but not completely. The compound also inhibited A23187-induced histamine release in a concentration-dependent manner with a minimal effective concentration of 25  $\mu$ M, although it was less than the effect on antigen. The compound inhibited A23187-induced tyrosine phosphorylation as well. Although also in this case the ~120-kDa proteins were sensitive to the compound, a substantial tyrosine phosphorylation of these proteins was still observed even when cells were treated with 50  $\mu$ M piceatannol (data not shown). Tyrphostins are the group of compounds that are known to inhibit epidermal growth factor (EGF) receptor kinase, p56<sup>lck</sup>, and platelet derived growth factor receptor kinase. AG99 (tyrphostin A46), a potent inhibitor of EGF receptor kinase (18) had little effect on histamine release and tyrosine phosphorylation induced by antigen (Fig. 3C). The compound had little effect on histamine release and tyrosine phosphorylation induced by A23187 (data not shown).

#### Effects of Tea Polyphenolic Compounds on Tyrosine Phosphorylation

We next examined the effect of tea polyphenolic compounds on tyrosine phosphorylation. Cells were incubated with increasing concentrations of EGCG for 30 min, and then stimulated with 1  $\mu$ g/ml antigen. EGCG inhibited antigen-induced tyrosine phosphorylation in a concentration-dependent manner (Fig. 4). The ~120-kDa proteins were also one of the most EGCG-sensitive molecules. Tyrosine phosphorylation of the ~120-kDa proteins was inhibited considerably by 50  $\mu$ M EGCG and almost completely inhibited by 200  $\mu$ M EGCG. Tyrosine phosphorylation of the 72-kDa proteins was also inhibited by EGCG. By contrast, EC had little effect on tyrosine phosphorylation of any proteins.

EGCG also inhibited A23187-induced tyrosine phosphorylation, although the effect was less than that on antigen-induced tyrosine phosphorylation (data not shown). Also in this case, the ~120-kDa proteins were one of the most EGCG-sensitive molecules. Tyrosine phosphorylation of the ~120-kDa proteins was almost



**FIG. 4.** Effects of EGCG and EC on tyrosine phosphorylation and histamine release induced by antigen. IgE-sensitized cells were incubated with EGCG or EC at the concentrations indicated at 37°C for 30 min and then stimulated with 1 μg/ml DNP-BSA for 5 min (for analysis of tyrosine phosphorylation) or for 30 min (for histamine measurement) at 37°C. The tyrosine-phosphorylated proteins from the cell lysates were analyzed by immunoblotting using anti-PY as described above. Histamine content of supernatants was determined by an ELISA method as described above. Arrow indicates the ~120-kDa proteins. %HR, percent histamine release. The results presented are representative of three different experiments.

completely inhibited by 200 μM EGCG. On the other hand, EC had little or rather stimulatory effect on the tyrosine phosphorylation.

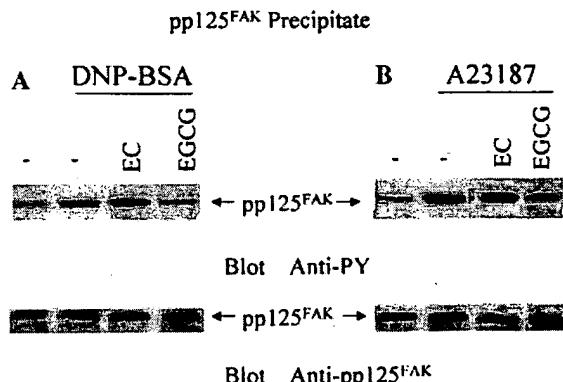
We noticed that treatment of RBL-2H3 cells with EGCG resulted in the change in cell morphology and increased resistance to the detachment from culture dishes by trypsin/EDTA treatment. Since EC did not exhibit such effect, the changes might be relevant to the inhibition of histamine release by EGCG. It has been shown that the focal adhesion kinase pp125<sup>FAK</sup> is involved in the control of cell-cell or cell-substrum adhesion (19, 20). pp125<sup>FAK</sup> also becomes phosphorylated on the tyrosine residues in response to the stimulation through Fc $\epsilon$ RI and is thought to play a role in the receptor-activated signal transduction (21, 22). Furthermore, the apparent molecular masses of the ~120-kDa proteins were similar to that of pp125<sup>FAK</sup>. Therefore, we next examined whether pp125<sup>FAK</sup> was one of the ~120-kDa proteins sensitive to EGCG. Cells were incubated with 200 μM EGCG or EC for 30 min, and stimulated with antigen or with A23187. Cell lysates were precipitated with specific antibody to pp125<sup>FAK</sup> and transferred to PVDF membranes, and tyrosine-phosphorylated proteins were analyzed by immunoblotting using the anti-phosphotyrosine mAb 4G10. Tyrosine phosphorylation of pp125<sup>FAK</sup> was usually low or under detectable levels in unstimulated cells. The tyrosine phosphorylation of pp125<sup>FAK</sup> was increased after stimulation with antigen (Fig. 5A) or with A23187 (Fig.

5B). EGCG inhibited tyrosine phosphorylation of pp125<sup>FAK</sup> induced by either stimulation, while EC had little effect (Fig. 5).

## DISCUSSION

We examined the effects of the two polyphenolic compounds EGCG and EC on histamine release induced by antigen or A23187. Despite their closed similarity in structure, EGCG could inhibit antigen-induced histamine release, while EC could not. The findings are consistent with those reported by Matsuo *et al.* (14, 15). A23187 has been shown to induce histamine release receptor-independently by increasing intracellular Ca<sup>2+</sup> levels. Thus, inhibition of histamine release by EGCG seems not to merely result from failure of binding between IgE-bound Fc $\epsilon$ RI and antigen and/or Ca<sup>2+</sup> influx. Rather EGCG seems to affect somewhat cellular events that occur in downstream of the calcium signal. In fact it has been shown that EGCG dose not affect Ca<sup>2+</sup> influx (15). In agreement with this previous report, our preliminary experiments using the calcium probe fura2 show that EGCG has little effect on the increase of intracellular Ca<sup>2+</sup> levels induced by antigen.

The novel finding in this study is that EGCG inhibits tyrosine phosphorylation of proteins induced by antigen and by A23187. Recent studies indicate that tyrosine phosphorylation of proteins is a critical event in the signaling pathways leading to secretion (5-7). In this study the role of tyrosine phosphorylation in the signaling was validated by using different types of tyrosine kinase inhibitors, piceatannol and AG99 (tyrphostin A46). Piceatannol has been shown to inhibit



**FIG. 5.** Effects of EGCG and EC on tyrosine phosphorylation of pp125<sup>FAK</sup> induced by antigen or by A23187. IgE-sensitized or IgE-untreated cells were incubated with 200 μM EGCG or EC at 37°C for 30 min, and then stimulated with 1 μg/ml DNP-BSA (A) or 2 μM A23187 (B) at 37°C for 5 min. Lysates were precipitated with antibody to pp125<sup>FAK</sup>, and the precipitates were immunoblotted with the anti-PY mAb 4G10 or anti-pp125<sup>FAK</sup> as described under Materials and Methods. The results presented are representative of three different experiments.

Syk kinase selectively (17). Consistent with this, the compound strongly prevented Fc $\epsilon$ RI-mediated tyrosine phosphorylation and histamine release where the kinase plays a pivotal role (9, 10). In addition, we noticed that piceatannol also inhibited A23187-induced tyrosine phosphorylation and histamine release significantly. The effect was specific for piceatannol because AG99 (tyrphostin A46), which inhibited EGF receptor tyrosine kinase preferentially, had little effect on these two events. Unlike antigen A23187 induced no substantial activation of Syk kinase in our cell system (data not shown). Therefore, EGCG seems to affect other tyrosine kinase(s) that lie(s) in downstream of  $\text{Ca}^{2+}$  influx. Tyrosine phosphorylation of the ~120-kDa proteins occurred in parallel with histamine release induced by antigen or by A23187, which was inhibited by EGCG but not inhibited by the inactive analog EC. Thus, the ~120-kDa proteins are likely to be one of such tyrosine kinases and/or their substrates in downstream of the calcium signal.

We also show that pp125<sup>FAK</sup> is one of the ~120-kDa proteins. Since morphology of RBL-2H3 cells altered considerably following treatment with EGCG but not with EC, EGCG might affect somewhat molecular machinery that is involved in the integrity of cytoskeleton, which plays a crucial role in cell degranulation. Thus, it is noteworthy that EGCG can affect pp125<sup>FAK</sup> tyrosine phosphorylation when considered with the fact that the molecule is profoundly involved in cell-cell or cell-substrum adhesion (19, 20). Furthermore, our finding is consistent with the notion that pp125<sup>FAK</sup> lies in the downstream of calcium signal (21) and plays a role in Fc $\epsilon$ RI-mediated histamine release (21, 22). Reconstitution experiments using the transfecting pp125<sup>FAK</sup> show that Fc $\epsilon$ RI-mediated histamine release correlates well with the expression levels of pp125<sup>FAK</sup> and that the stable expression of the molecule enhances the response (22). Thus, inhibition of pp125<sup>FAK</sup> tyrosine phosphorylation is a likely mechanism of the inhibitory effect of EGCG on receptor-dependent secretion. On the other hand, no correlation has been found between pp125<sup>FAK</sup> tyrosine phosphorylation and A23187-induced histamine release (22). Therefore, the ~120-kDa proteins except pp125<sup>FAK</sup> or other proteins might be involved in the inhibition of A23187-induced secretion by EGCG.

In conclusion, here we show that EGCG can inhibit histamine release from RBL-2H3 cells mainly by inhibiting tyrosine phosphorylation, a critical event in the signal transduction leading to secretion. These find-

ings suggest that some tea polyphenolic compounds potentially serve as effective therapeutic tools for allergic diseases.

#### ACKNOWLEDGMENTS

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## Inhibitory Effects of Apple Polyphenol on Induced Histamine Release from RBL-2H3 Cells and Rat Mast Cells

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The anti-allergic activities of polyphenol fractions extracted from immature fruits of apple (*Rosaceae, Malus sp.*) were evaluated by *in vitro* assays. A crude apple polyphenol (CAP) fraction, which had been obtained from the juice of immature apples by reverse-phase column chromatography, was further purified by LH-20 column chromatography to obtain an apple condensed tannin (ACT) fraction consisting of linear oligomeric epicatechins from the dimer to pentadecamer. ACT strongly inhibited the release of histamine from rat basophilic leukemia (RBL-2H3) cells stimulated by the antigen-stimulation and from rat peritoneal mast cells stimulated by compound 48/80. The IC<sub>50</sub> values for histamine release were 30 µg/ml and 25 µg/ml, respectively. ACT also inhibited hyaluronidase activity and the increase in intracellular free calcium concentration in RBL-2H3 cells stimulated with the antigen. These results suggest that ACT affected early signal transduction including the calcium influx.

**Key words:** apple polyphenol; anti-allergic activity; histamine release; hyaluronidase; calcium influx

It is well-known that apple contains several classes of polyphenols, namely simple polyphenols such as chlorogenic acid, (+)-catechin, (-)-epicatechin, phloridzin, rutin and other flavonoids,<sup>1-3)</sup> and condensed tannins such as procyanidin B1, B2 and C1.<sup>4-7)</sup> The composition of these polyphenols affects the taste,<sup>8)</sup> flavor and color characteristics of apple products.<sup>9,10)</sup> Burda *et al.* and Murata *et al.* have reported that the content of polyphenols in immature apples was three to ten times higher than that of mature fruits and decreased with progressive maturity from flowering.<sup>11,12)</sup> Since their taste is poor, immature apples have generally been treated as waste. In order to develop a method for utilizing immature apples, we have investigated the properties and biological activities of their constituents.<sup>13,14)</sup> In the present study, we investigate the inhibitory effects of the condensed tannin fraction and of some simple polyphenols extracted from immature apples on the induced histamine release from RBL-2H3 cells and rat mast cells to evaluate their anti-allergic activities, the release of histamine from basophils or mast cells being an essential

step in an immediate type of allergic reaction. We also evaluate the inhibitory effects on the activity of hyaluronidase which is one of the lysosomal enzymes<sup>15)</sup> and is released with chemical mediators from mast cells or basophils. Some authors have reported that simple polyphenols had anti-allergic activities.<sup>16-21)</sup> However, few data have been presented regarding the activities of condensed tannin.<sup>16)</sup> So we examined the effect of the condensed tannin fraction on the increase in intracellular free calcium concentration to investigate the inhibitory mechanism.

### Material and Method

**Sample preparation.** Crude apple polyphenol (CAP) and apple condensed tannin (ACT) were obtained from immature apples (*Rosaceae Malus pumila* cv. *Fuji*) harvested from May to June in the Aomori Prefecture by crushing in the presence of sulfuric acid (0.05%) and then pressing. The obtained homogenate was clarified by centrifugation at 3500 g and passed through a glass filter to afford clear juice. This clear juice was applied to a Sepabeads SP-850 column (Mitsubishi Kagaku Co.). After washing the column with deionized water, the 65% ethanolic eluate was collected, evaporated and lyophilized to obtain CAP. CAP was then chromatographed in a Sephadex LH-20 column (Pharmacia Biotech. Co.). The column was washed with deionized water and 60% ethanol to remove the simple polyphenols and then eluted with 70% acetone. The resulting eluate was evaporated and lyophilized to obtain ACT. An HPLC analysis revealed that CAP contained procyanidin B1 (3%), (+)-catechin (0.5%), procyanidin B2 (7%), chlorogenic acid (20%), caffeic acid (3%), (-)-epicatechin (6%), *p*-coumaric acid (1%), phloridzin (4%), phloretin (1%), ACT (40%) and unknown residual components (15%). The structures of these compounds are shown in Fig. 1.

**Reagents.** Disodium chromoglycate (DSCG), traniast and ketotifen fumarate were purchased from Fujisawa Yakuhin Kogyo Co., Kissei Yakuhin Kogyo Co. and Sankyo Co., respectively. Chlorogenic acid and (-)-epicatechin, which had been obtained from Wako Pure Chemical Co., and phloridzin, obtained from Tokyo

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**Abbreviations:** RBL, rat basophilic leukemia; CAP, crude apple polyphenol; ACT, apple condensed tannin; DSCG, disodium chromoglycate; TNP, trinitrophenol; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

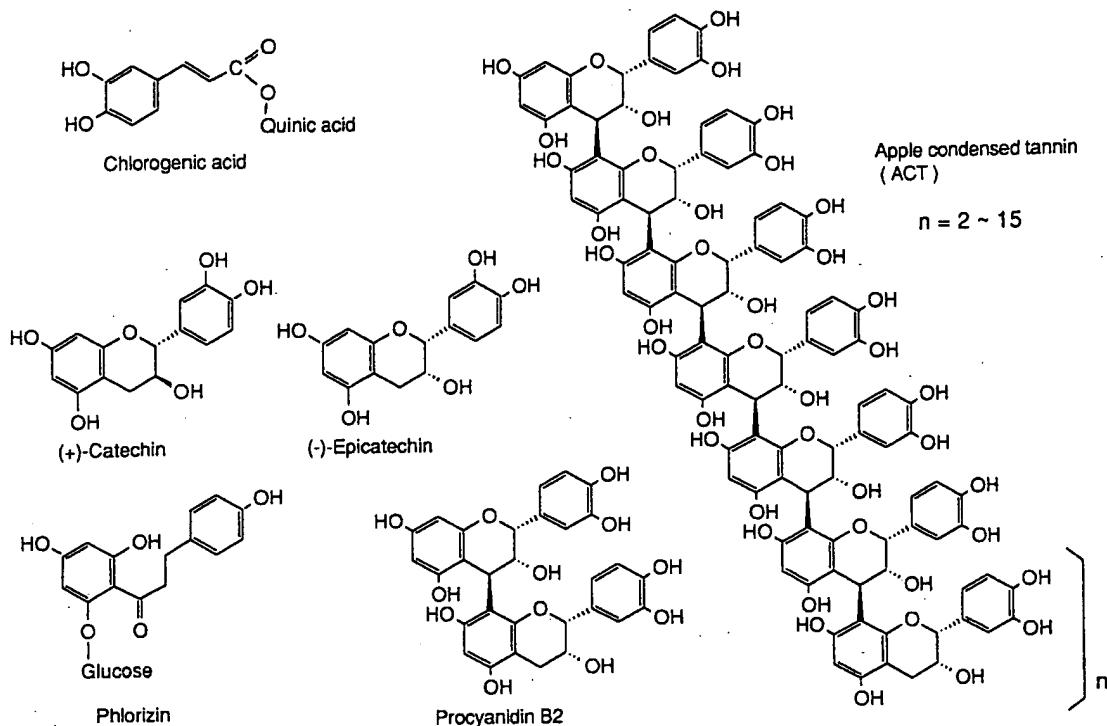


Fig. 1. Chemical Structures of the Main Compounds in Crude Apple Polyphenol (CAP).

CAP was obtained from the juice of immature apples (*Rosaceae Malus sp.*) by column chromatography and contained many polyphenolic compounds. ACT is the fraction of oligomeric procyandins containing the dimer to pentadecamer of epicatechin as an unit.<sup>13</sup>

Kasei Co., were used for the *in vitro* assays. Fura-2-AM was obtained from Dojindo Co. (Kumamoto, Japan).

**Cells.** RBL-2H3 cells (JCRB 0023) were obtained from the Japan Cancer Research Resources Bank. The cells were maintained in an alpha minimum essential medium ( $\alpha$ -MEM; Gibco Brl Co.) supplemented with 10% fetal bovine serum (FBS; Hyclone Co.) and 0.08 mg/ml of kanamycin (Nakarai Chemical Co.). The monoclonal anti-TNP IgE antibody producing hybridoma (IGELa2) was purchased from ATCC (TIB 142).

**Assay of the inhibitory effect on histamine release from RBL-2H3 cells.** The assay of the inhibitory effect on histamine release from RBL-2H3 cells was performed according to the method described by Kawasaki *et al.*<sup>22</sup> with some modifications. Test samples were added at two stages, the IgE sensitization stage and the antigen stimulation stage. Cells ( $1 \times 10^5$  cells/well) were precultured at 37°C for 24 h in a 24-well flat-bottomed microtiter plate in 0.5 ml per well of the medium. The supernatants were discarded and the cells were incubated at 37°C for 120 min with  $\alpha$ -MEM containing 2% FBS and anti-TNP IgE (1000-fold diluted ascites) [at the IgE sensitization stage, a test sample was added to the medium and not the HEPES buffer]. The cells were washed three times with the HEPES buffer (140 mM NaCl, 5 mM KCl, 0.6 mM MgCl<sub>2</sub>, 1.0 mM CaCl<sub>2</sub>, 5.5 mM glucose, 0.1% bovine serum albumin (BSA) and 5 mM HEPES

(n-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid) at pH 7.4] to eliminate free IgE. After incubating at 37°C for 10 min in 0.4 ml of buffer containing a test sample [antigen stimulation stage], the cells were challenged with DNP (dinitrophenyl)-BSA (0.1 ml of 100  $\mu$ g/ml in the buffer) at 37°C for 35 min. The plate was put on ice, and the cold HEPES buffer (0.6 ml) was added to each well to stop the reaction. The supernatant was taken out from each well, and 20  $\mu$ l of perchloric acid was added. After centrifuging at 12,000 rpm for 30 min, each supernatant was passed through a 0.45- $\mu$ m filter. The amount of histamine in each filtrate was analyzed by HPLC according to the post column conversion method by using *o*-phthalaldehyde.<sup>22</sup>

**Assay of the inhibitory effect on histamine release from rat peritoneal mast cells.** Preparation of the mast cells and the assay of the inhibitory effect on histamine release from rat peritoneal mast cells were based on the method of Hirai *et al.*<sup>23</sup> Seven-week-old male Wistar rats (150 g mean weight) were purchased from Japan SLC (Hamamatsu, Japan). Rat peritoneal mast cells were collected from the rat peritoneal cavity after injecting a Tyrode solution and then purified by density-gradient centrifugation with Percoll (Pharmacia LKB Biotechnology, Uppsala, Sweden).

A test sample was added to the cell suspension after preincubating at 37°C for 10 min then stimulated with compound 48/80 for 15 min. The amount of histamine

in the solution was analyzed by the method already described.

**Assay of hyaluronidase.** Hyaluronidase (bovine type) was purchased from Sigma Chemical Co., its specific activity being 800 NF unit/mg of protein. Hyaluronidase activity was determined according to the Morgan-Elson method<sup>24</sup> with some modifications. The mixture (0.3 ml) containing a 0.1 M acetate buffer (adjusted to pH 4.0 by HCl), the test sample and hyaluronidase (340 NF unit) was preincubated at 37°C for 20 min. Then, 0.2 ml of 0.5 mg/ml of a solution of compound 48/80 (as a hyaluronidase activator; Sigma Chemical Co.) was added to the mixture, and the whole incubated at 37°C for 20 min. The reaction was initiated by adding 0.5 ml of 1.2 mg/ml of a solution of the hyaluronic acid potassium salt (Wako Pure Chemical Co.), carried out at 37°C for 40 min and then terminated by adding 0.4 N NaOH (0.2 ml). A 0.2-ml sample of a 0.8 M sodium borate solution (pH 9.1) was then added to the reaction mixture that had been precooled in an ice bath. The mixture was then boiled at 100 for 3 min and cooled in the ice bath again. After adding 6 ml of a *p*-diethylaminobenzaldehyde (*p*-DAB) reagent, the mixture was incubated at 37°C for 20 min. Finally, the optical density at 585 nm was measured as a parameter of the hyaluronidase activity.

**Monitoring calcium influx with a fluorospectrophotometer.** Calcium influx was monitored by the method of R. Teshima *et al.*<sup>25</sup> with some modifications. RBL-2H3 cells were suspended in a PIPES buffer (140 mM NaCl, 5 mM KCl, 0.6 mM MgCl<sub>2</sub>, 1.0 mM CaCl<sub>2</sub>, 5.5 mM glucose, 0.1% BSA and 10 mM PIPES (piperazine-N,N'-bis[2-ethanesulfonic acid])) at pH 7.4) containing 6 μM fura-2-AM and anti-TNP IgE for 15 min at 37°C. The cells were washed three times with the buffer, resuspended in a fresh buffer in a quartz cuvette and continuously stirred at 37°C with a magnetic stirrer (Shimadzu RF-5000). ACT was added to the cuvette with a microsyringe. After incubating for 5 min at 37°C, the cells were challenged with DNP-BSA. The intracellular free calcium ion concentration was monitored as the ratio of fluorescence excited at two wavelengths, 335 and 362 nm. The emission wavelength was 495 nm.

## Results

### Inhibitory effects on histamine release from RBL-2H3 cells stimulated by antigen

A test sample is normally added to a buffer solution containing cells challenged with the antigen (DNP-BSA). In this case, it is thought that the test sample has an effect downstream from the antigen-stimulated stage in the cascade of an immediate type of allergic reaction. The inhibitory effects of CAP, (-)-epicatechin, chlorogenic acid, phloridzin and ACT at a concentration of 100 μg/ml and of ketotifen fumarate at a concentration of 20 μg/ml are shown in Fig. 2, their inhibition being 70%, 21%, 3%, 8% and 98%, and 42%, respectively. The assay of ketotifen, which is an anti-allergic drug

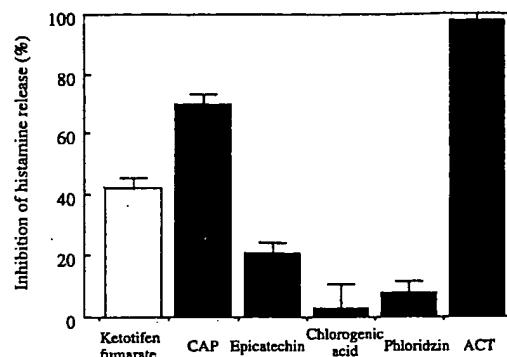


Fig. 2. Inhibitory Activity of Apple Polyphenol on Histamine Release from RBL-2H3 Cells.

RBL-2H3 cells were precultured for 24 h in a 24-well microtiter plate. The cells were incubated for 120 min with α-MEM containing 2% FBS and anti-TNP IgE (1000-fold diluted ascites). After washing with a HEPES buffer, the cells were incubated for 10 min with the buffer containing a test sample, and then challenged with DNP-BSA (20 μg/ml) for 35 min. The plate was put on ice, and the cold HEPES buffer was added to each well to stop the reaction. The analysis of histamine is described in the Materials and Methods section. The concentrations of CAP, (+)-epicatechin, chlorogenic acid, phloridzin and ACT were each 100 μg/ml (these polyphenols were not cytotoxic at this concentration) in the buffer. Ketotifen fumarate, an anti-allergic drug, was used as a positive control at 20 μg/ml because of its cytotoxicity. Each value is the average of triplicate cultures, and each bar indicates the mean ± SE (n=3).

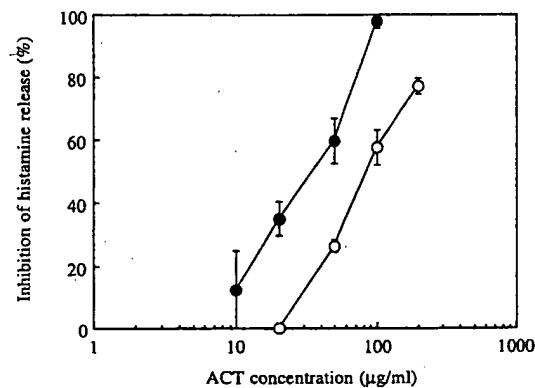


Fig. 3. Dose Dependency of ACT on the Inhibition of Histamine Release at the IgE Sensitization Stage and Antigen Stimulation Stage.

At the IgE sensitization stage, ACT was added in α-MEM with IgE (○), the IC<sub>50</sub> value being 95 μg/ml. And at the antigen stimulation stage, ACT was added in the HEPES buffer with preincubation and with DNP-BSA stimulation (●), the IC<sub>50</sub> value being 30 μg/ml. Each value is the average of triplicate cultures, and each bar indicates the mean ± SE (n=3).

and was used as a positive control, could not be carried out at the concentration of 100 μg/ml because of its cytotoxicity. The dose dependency of ACT on the inhibition is shown in Fig. 3, the IC<sub>50</sub> value being 30 μg/ml. At 20 μg/ml, the inhibition by ACT (35%) was almost as strong as that by ketotifen fumarate (41%).

In order to obtain more information about the anti-allergic effects of ACT, an assay in which ACT was added at the IgE sensitization stage was carried out. As shown in Fig. 3, ACT also acted at this stage, although the inhibition of histamine release was lower (95  $\mu\text{g}/\text{ml}$ ) than that at the antigen stimulation stage.

*Inhibitory effects on histamine release from rat peritoneal mast cells stimulated by compound 48/80*

Since RBL-2H3 cells are basophilic tumor cells, they are somewhat different from mast cells.<sup>26</sup> Therefore, we investigated the effects of the polyphenols in apple on the histamine release from rat peritoneal mast cells that had been stimulated by compound 48/80. The inhibitory effects of CAP, (-)-epicatechin, chlorogenic acid, phloridzin, ACT and ketotifen fumarate at a concentration of 100  $\mu\text{g}/\text{ml}$  are shown in Fig. 4, their inhibition being 33%, 8%, 7%, 0%, 93% and 74%, respectively. ACT significantly inhibited histamine release more strongly than ketotifen did. The dose dependency of ACT on the inhibition is shown in Fig. 5, the  $\text{IC}_{50}$  value being 25  $\mu\text{g}/\text{ml}$ .

*Inhibitory effects on hyaluronidase activity*

The  $\text{IC}_{50}$  values for CAP, (-)-epicatechin, chlorogenic acid, phloridzin, ACT, tranilast and DSCG were 260  $\mu\text{g}/\text{ml}$ , 300  $\mu\text{g}/\text{ml}$ , 260  $\mu\text{g}/\text{ml}$ , >500  $\mu\text{g}/\text{ml}$ , 80  $\mu\text{g}/\text{ml}$ , >500  $\mu\text{g}/\text{ml}$  and 80  $\mu\text{g}/\text{ml}$ , respectively. ACT inhibited hyaluronidase activity more strongly than the simple polyphenols tested and was as strong as DSCG.

*Effect of ACT on the calcium influx of RBL-2H3 cells*

To investigate the inhibitory mechanism of ACT on histamine release from RBL-2H3 cells, the effect of

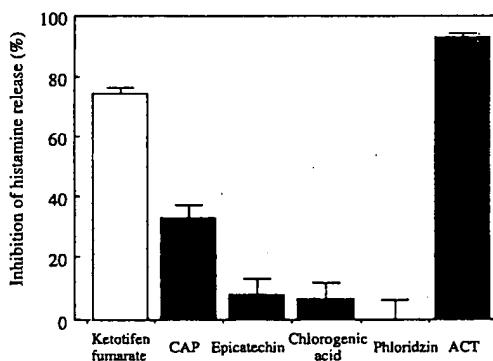


Fig. 4. Inhibitory Activity of Apple Polyphenol on Histamine Release from Rat Peritoneal Mast Cells.

Rat peritoneal mast cells were collected from the peritoneal cavity of male Wistar rat after injecting a Tyrode solution and were purified by density-gradient centrifugation with Percoll. A test sample was added to the cell suspension after preincubating at 37°C for 10 min and then stimulated with compound 48/80 (5  $\mu\text{g}/\text{ml}$ ) for 15 min. The analysis of histamine is described in the Materials and Methods section. Each value is the average of triplicate cultures, and each bar indicates the mean  $\pm$  SE ( $n=3$ ). The concentrations of CAP, (+)-epicatechin, chlorogenic acid, phloridzin and ACT were each 100  $\mu\text{g}/\text{ml}$  in the buffer. Ketotifen fumarate, an anti-allergic drug that was used as a positive control, was 100  $\mu\text{g}/\text{ml}$ .

ACT on the calcium influx of the cells was examined with a fluorospectrophotometer. As shown in Fig. 6, after stimulation with the antigen, calcium influx started and the intracellular calcium ion concentration reached a plateau 120 sec later. When the cells had been preincubated with ACT before antigen stimulation, the calcium influx was inhibited, the inhibitory effect of ACT showing dose-dependency.

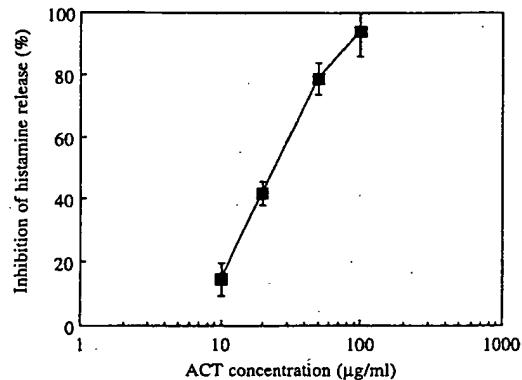


Fig. 5. Dose Dependency of ACT on the Inhibition of Histamine Release from Rat Peritoneal Mast Cells Induced with Compound 48/80.

Rat peritoneal mast cells were collected from the peritoneal cavity of male Wistar rat and purified by density-gradient centrifugation with Percoll. ACT was added to the cell suspension after preincubating at 37°C for 10 min and then stimulated with compound 48/80 (5  $\mu\text{g}/\text{ml}$ ) for 15 min. The analysis of histamine is described in the Materials and Methods section. The  $\text{IC}_{50}$  value was 25  $\mu\text{g}/\text{ml}$ . Each value is the average of triplicate cultures, and each bar indicates the mean  $\pm$  SE ( $n=3$ ).

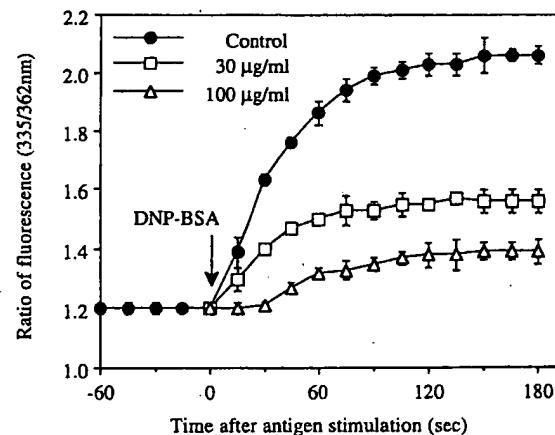


Fig. 6. Effect of ACT on the Calcium Influx in RBL-2H3 Cells.

RBL-2H3 cells were loaded with fura-2-AM and sensitized with anti-TNP IgE in a PIPES buffer for 15 min at 37°C. After washing, the sensitized cells were resuspended in the buffer in a quartz cuvette and preincubated with 30  $\mu\text{g}/\text{ml}$  (□) or 100  $\mu\text{g}/\text{ml}$  (△) of ACT for 5 min at 37°C. The cells were then stimulated with DNP-BSA. The intracellular calcium ion concentration was monitored as the ratio of fluorescence excited at the two wavelengths of 335 and 362 nm. The emission wavelength was 495 nm.

## Discussion

In our earlier study, MALDI-TOF-MS and FAB-MS analyses revealed that ACT consisted of oligomeric catechins (procyanidins) from the dimer to pentadecamer.<sup>13</sup> In this study, ACT more strongly inhibited the release of histamine from both RBL-2H3 cells and rat peritoneal mast cells than CAP did. On the contrary, chlorogenic acid, (-)-epicatechin and phloridzin, which are simple polyphenols and the main components of CAP other than ACT, each showed less inhibitory effect than that of CAP. These facts suggest that the inhibitory activity of CAP against histamine release was mostly due to ACT. Shinohara *et al.* have reported that the polyphenol-rich fractions from apple showed anti-mutagenic activity.<sup>27</sup> Several authors have reported that oligomeric catechins (proanthocyanidin) from *Areca catechu*<sup>6</sup> and *Vitis vinifera*<sup>7</sup> showed anti-oxidative activities<sup>28-30</sup> and inhibited platelet aggregation and arachidonate metabolism.<sup>31</sup> However, no one has so far reported that oligomeric catechins could inhibit histamine release.

Zhu *et al.* have recently reported that polyphenols would inhibit the binding of specific radio-ligands to various receptors.<sup>32</sup> In the immediate type of allergic reaction cascade, IgE sensitizes basophils or mast cells which have a specific Fc receptor for IgE on the cell-surface. Therefore, it is of interest whether ACT would have an effect at the IgE sensitization stage in the cascade and inhibit histamine release. Fig. 3 suggests that ACT had an effect not only downstream from the antigen stimulation stage but also at the IgE sensitization stage. Since the antigen-antibody reaction was sensitive to pH in the buffer solution, the effect of ACT on pH was examined. As shown in the Table, ACT hardly changed the pH value of the buffer solution, suggesting that the inhibitory activity of ACT was not due to any pH change in the buffer solution.

It is believed that histamine is released from the intracellular secretory granules induced by an increase in the intracellular calcium ion concentration and by activation of the signal transduction system.<sup>33-35</sup> As shown in Fig. 6, ACT inhibited calcium influx. This result strongly suggests that ACT affected the signal transduction system and lead to the elevation in intracellular calcium ion concentration.

Tea polyphenol also inhibited histamine release from RBL-2H3 cells after antigen stimulation. However, it is thought that the mechanism for the inhibitory effect of tea polyphenol was different from that of ACT because epigallocatechin gallate did not inhibit the increase of intracellular calcium ion concentration in RBL-2H3.<sup>36</sup>

The release of histamine from RBL-2H3 cells by antigen stimulation and that from rat peritoneal mast cells by compound 48/80 are thought to occur by different mechanisms. The former is concerned with calcium influx from an outercellular solution, but the latter is not. Our results that ACT strongly inhibited the release of histamine from RBL-2H3 cells at the IgE sensitization stage and downstream from the antigen-stimulated stage, and the release of histamine from rat peritoneal

Table pH Value of the HEPES Buffer\* Solution Containing ACT at 37°C

Concentration of ACT ( $\mu$ g/ml)	pH Value
None	7.38
10	7.39
20	7.39
50	7.39
100	7.38
200	7.35

\* The buffer consisted of 140 mM NaCl, 5 mM KCl, 0.6 mM MgCl<sub>2</sub>, 1.0 mM CaCl<sub>2</sub>, 5.5 mM glucose, 0.1% BSA and 5 mM HEPES, and pH was adjusted to 7.4.

mast cells might suggest that ACT acted as a multi-functional inhibitor against histamine release.

It has been reported that hyaluronidase played an important role in the allergic action, because some anti-allergic drugs inhibited hyaluronidase. In this study, ACT inhibited hyaluronidase as strongly as DSCG did, which is one of the strongest inhibitors of hyaluronidase and is clinically used as an anti-allergic drug. This result suggests that ACT would exhibit anti-allergenic activity not only as an inhibitor of histamine release but also as an inhibitor of the enzyme which increases cell permeability.

Hackett *et al.* have reported that 55% of (+)-catechin was excreted in urine, 90% of that being excreted within 24 h by humans following an oral administration.<sup>37</sup> The absorption, distribution and excretion of ACT (including oligomers of catechins) have not yet been studied.

In conclusion, ACT inhibited the release of histamine from RBL-2H3 cells and from rat mast cells and the hyaluronidase activity as strongly as anti-allergic drugs did. These data suggest that ACT may possess anti-allergic activity to humans. The further fractionation of ACT and evaluation of the inhibitory effect of each fraction on histamine release are in progress. Future studies may clarify whether the anti-allergic activities increase with increasing number of catechin units.

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# Inhibition of mitochondrial proton F0F1-ATPase/ATP synthase by polyphenolic phytochemicals

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1 Mitochondrial proton F0F1-ATPase/ATP synthase synthesizes ATP during oxidative phosphorylation. In this study, we examined the effects of several groups of polyphenolic phytochemicals on the activity of the enzyme.

2 Resveratrol, a stilbene phytoalexin that is present in grapes and red wine, concentration-dependently inhibited the enzymatic activity of both rat brain and liver F0F1-ATPase/ATP synthase ( $IC_{50}$  of 12–28  $\mu$ M).

3 Screening of other polyphenolic phytochemicals using rat brain F0F1-ATPase activity resulted in the following ranking potency ( $IC_{50}$  in parenthesis): piceatannol (8  $\mu$ M) > resveratrol (19  $\mu$ M) = (–)epigallocatechin gallate (17  $\mu$ M) > (–)epicatechin gallate, curcumin (45  $\mu$ M) > genistein = biochanin A = quercetin = kaempferol = morin (55–65  $\mu$ M) > phloretin = apigenin = daidzein (approx. 100  $\mu$ M). Genistin, quercitrin, phloridzin, (+)catechin, (+)epicatechin, (–)epicatechin and (–)epigallocatechin had little effect at similar concentrations. Tannic acid, theaflavins (tea extract) and grape seed proanthocyanidin extract (GSPE) had  $IC_{50}$  values of 5, 20 and 30  $\mu$ g ml<sup>–1</sup>, respectively. Several monophenolic antioxidants and non-phenolic compounds were ineffective at concentrations of 210  $\mu$ M or higher.

4 The inhibition of F0F1-ATPase by resveratrol and genistein was non-competitive in nature.

5 The effects of polyphenolic phytochemicals were additive.

6 Both resveratrol and genistein had little effect on the  $Na^+/K^+$ -ATPase activity of porcine cerebral cortex, whereas quercetin had similar inhibitory potency as for F0F1-ATPase.

7 In conclusion, the ATP synthase is a target for dietary phytochemicals. This pharmacological property of these phytochemicals should be included in the examination of their health benefits as well as potential cytotoxicity.

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**Keywords:** Phytoestrogens; F0F1-ATPase/ATP synthase;  $Na^+/K^+$ -ATPase; flavonoids; mitochondria; resveratrol; genistein; catechins; tea

**Abbreviations:** AEBSF, 4-(2-aminoethyl)-benzenesulphonyl fluoride; CHAPS, (3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulphonate; c.i., confidence interval; ECG, (–)epicatechin gallate; EGCG, (–)epigallocatechin gallate; GSPE, grape seed proanthocyanidin extract; I3C, indole-3-carbinol;  $K_m$ , Michaelis constant; OPC, oligomeric proanthocyanidin complex; OSCP, oligomycin sensitivity-conferring protein;  $P_i$ , inorganic phosphate;  $V_{max}$ , maximal specific activity

## Introduction

F0F1-ATPase/ATP synthase (F-type ATPase, complex V) is present in the inner membrane of eukaryotic mitochondria and acts as the powerhouse of the cell by synthesizing ATP. It can also operate in the reverse direction, hydrolysing ATP and pumping protons under certain conditions. The enzyme can be separated into two major complexes: F1 and F0 (Pedersen & Amzel, 1993; Boyer, 1997). F1 is a water-soluble catalytic complex consisting of five subunits ( $\alpha\beta\gamma\delta\epsilon$ ), with the catalytic site located on the  $\beta$  subunit. F0 is made up of several membrane proteins (a, b, c, d, e, F6, A6L) and oligomycin sensitivity-conferring protein (OSCP), which contributes to the stalk region between F0 and F1. In addition, a native peptide is also bound to the F1 under de-energized conditions that serves to inhibit the ATPase activity of the enzyme (called F1 inhibitor protein, IF1).

Recent studies suggest that both  $\alpha$  and  $\beta$  subunits of the F1-ATPase may be present on the surface of human umbilical vein endothelial cells and are binding sites for angiostatin, a proteolytic fragment of plasminogen that is both a potent antagonist of angiogenesis and an inhibitor of tumour growth (Moser *et al.*, 1999). Antibodies against  $\alpha$  subunit labelled the cell surface and inhibited angiostatin's anti-proliferative effect on endothelial cells. Earlier,  $\beta$  subunit had been localized to the cell surface of three human tumour cell lines (erythroleukaemia K562 cells, lung adenocarcinoma A549 cells and Burkitt's lymphoma Raji cells), but not to that of normal human erythrocytes or lymphocytes (Das *et al.*, 1994). The surface  $\beta$  subunit in tumour cells induces lymphocyte-mediated cell killing, whereas antibodies against the  $\beta$  subunit inhibit this cytotoxicity. These studies suggest the key involvement of cell surface F-ATPase subunits in tumour growth and inhibition, which could be potential therapeutic targets for cancers.

Several inhibitors of ATP synthase have been described, including efrapeptin, oligomycin, aurovertin B and azide (Linnett & Beechey, 1979). The binding pocket for the F1-

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targeting inhibitor efrapeptin is localized in  $\alpha$ ,  $\beta$  and  $\gamma$  subunits (Abrahams *et al.*, 1996) while that for aurovertin B is localized mainly in the  $\beta$  subunit (van Raaij *et al.*, 1996). Inhibition of F0F1-ATPase/ATP synthase by the F0-targeting inhibitor, oligomycin, has been shown to protect or postpone cell injury by preserving ATP during ischaemia (Vuorinen *et al.*, 1995) and to induce apoptosis in tumour cells (Wolvetang *et al.*, 1994). Oligomycin also inhibits the apoptosis induced by Bax, a pro-apoptotic protein localized on the mitochondrial outer membrane, and F0F1-ATPase is required for the killing of cells by Bax (Matsuyama *et al.*, 1998).

We recently reported that rat brain F0F1-ATPase was retained by oestradiol affinity columns and that oestradiol and several other oestrogens inhibited its activity (Zheng & Ramirez, 1999a,b). Earlier, diethylstilbestrol, a synthetic oestrogen, was shown to inhibit rat liver F0F1-ATPase (McEnery & Pedersen, 1986; McEnery *et al.*, 1989). Although these effects require pharmacological (micromolar) concentrations of these oestrogens, which may not be physiologically relevant, phytoestrogens and other structurally similar polyphenolic phytochemicals are present in abundance in human food (Dewick, 1997; Bravo, 1998). It is worth mentioning that one group of weak oestrogenic phytochemicals—flavones, such as quercetin—has been shown to inhibit bovine and porcine heart F0F1-ATPase (Lang & Racker, 1974; Di Pietro *et al.*, 1975). Quercetin also inhibits a number of other ATPases, such as  $\text{Na}^+/\text{K}^+$ -ATPase,  $\text{Ca}^{2+}$ -ATPase and more than a dozen

other enzymes (Fewtrell & Gomperts, 1977; Ferrell *et al.*, 1979; Hirano *et al.*, 1989; McKenna *et al.*, 1996).

In this study, we report on the effects of several groups of polyphenolic phytochemicals that have been shown to be important in human diets on the activity of mitochondrial F0F1-ATPase/ATP synthase, including stilbenes, isoflavones, flavones, catechins, chalcones and several others (Figure 1). In addition, we report the kinetic mechanisms of inhibition for selected compounds as well as their effect on  $\text{Na}^+/\text{K}^+$ -ATPase. Many of these phytochemicals exhibit diverse activities such as antioxidant, cardiovascular protective, anti-osteoporotic, cancer chemopreventative, anti-mitotic and oestrogenic actions (Frankel *et al.*, 1993; Adlercreutz, 1995; Gehm *et al.*, 1997; Jang *et al.*, 1997; Hsieh *et al.*, 1998; Dashwood, 1998; Setchell, 1998; Murkies *et al.*, 1998; Tham *et al.*, 1998; Hollman *et al.*, 1999; Yang *et al.*, 1999; Ye *et al.*, 1999). Our study, therefore, could provide a potential mechanism for the actions of phytochemicals through targeting of the F0F1-ATPase/ATP synthase.

## Methods

### Materials

4-(2-Aminoethyl)-benzenesulphonyl fluoride (AEBSF), an irreversible serine protease inhibitor, was from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Oligomycin (mixture of

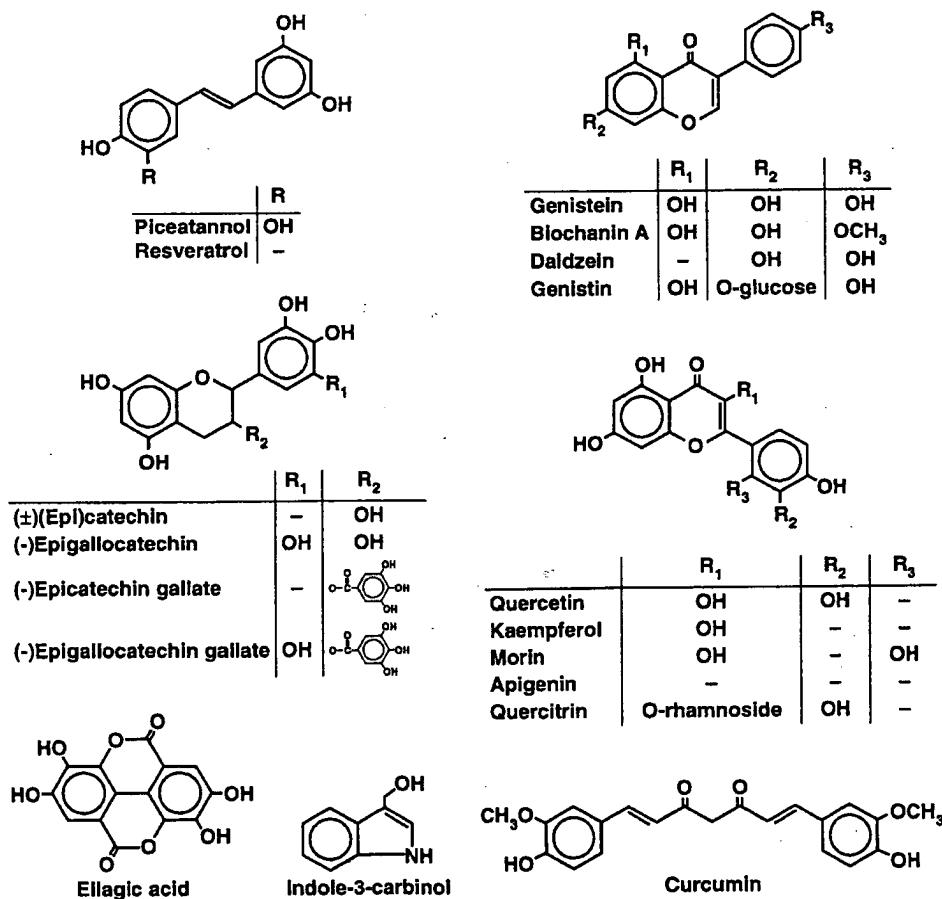


Figure 1 Structures of selected phytochemicals tested for their inhibitory effects on the rat mitochondrial F0F1-ATPase/ATP synthase activity. Many have been shown to be phytoestrogens, such as stilbenes, isoflavones and flavones.

A, B and C) was purchased from Aldrich (Milwaukee, WI, U.S.A.) and prepared in methanol as a stock solution ( $1 \mu\text{g } \mu\text{l}^{-1}$ ). Efrapeptin (efrastatin, A23871) was kindly provided by Dr J. Clemens (Eli Lilly & Co., Indianapolis, IN, U.S.A.) and dissolved in sterile distilled water at a concentration of 0.1 mM. IH636 Grape seed proanthocyanidin (GSPE) was kindly provided by Dr. D. Bagchi (InterHealth Nutraceuticals, Concord, CA, U.S.A.) as a mixture of proanthocyanidins (>54% dimeric, 13% trimeric, 6.8% tetrameric and small amount of monomeric and other high molecular weight oligomeric proanthocyanidin complexes [OPC]). All other chemicals and reagents, including porcine cortex  $\text{Na}^+/\text{K}^+$ -ATPase were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Ouabain (stock solution of  $5 \mu\text{g } \mu\text{l}^{-1}$ ) was dissolved in sterile distilled water. Phytochemicals and diethylstilbestrol were usually prepared in 100% ethanol with a final concentration of 10 mM or above, except for daidzein, phloridzin and GSPE, which were prepared in 50% ethanol/50% dimethyl sulphoxide. The final solvent concentrations in the reaction solution were usually 0.7% and occasionally 1.4%. The amount of solvents had minimal effect on the responses (<5%). Nevertheless, in every experiment, control trials were performed with the same amount of vehicle included.

#### Animals

Adult female Sprague-Dawley rats (60–120 days old) were maintained on a 14:10 h light/dark cycle (lights on at 0700 h) with food and water available *ad libitum*. Animals were taken care of in accordance with federal and institutional guidelines and killed by rapid decapitation.

#### Preparation of mitochondria

Mitochondria fractions from whole brain and liver of adult Sprague-Dawley female rats (unknown oestrous cycle) were prepared in Tris buffer (mM: Tris-HCl 50, NaCl 120, KCl 5,  $\text{MgSO}_4$  1,  $\text{CaCl}_2$  1, 10% glycerol, AEBSF 0.5, bacitracin 0.1; pH 7.4 at 4°C), as described previously (Zheng & Ramirez, 1999a). Briefly, tissues were homogenized in Tris buffer (10 ml per g tissue) with a Teflon glass homogenizer (10 strokes in 1 min). Homogenates were centrifuged at  $600 \times g$  for 10 min and the resulting supernatant was centrifuged again at  $15,000 \times g$  for 5 min to precipitate the mitochondrial fraction. Samples were assayed for protein concentration by the method of Bradford (1976), using bovine serum albumin as the standard, and either kept at 4°C for use the same day or stored at –80°C.

#### Solubilization

Mitochondrial fractions were solubilized in Tris buffer containing 1%  $\text{w v}^{-1}$  digitonin (Zheng & Ramirez, 1999a) or 1% 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propane sulphonate (CHAPS) at 4°C as described (McEnery *et al.*, 1984). Solubilization allows the exposure of F0F1-ATPase, which is not accessible to many reagents in a coupled assay (see below), in intact mitochondria.

#### Preparation of submitochondrial particles

Submitochondrial particles were prepared by sonication as described (Ragan *et al.*, 1987). Briefly, freshly prepared brain mitochondrial fractions were sonicated for  $6 \times 10$  s and centrifuged at  $15,000 \times g$  for 5 min at 4°C. The supernatant

was centrifuged again at  $125,000 \times g$  for 60 min; the resulting pellet (submitochondrial particles) was resuspended in Tris buffer and used within 24 h.

#### Assay for ATPase activity

The mitochondrial F0F1-ATPase activity was measured spectrophotometrically at 340 nm by coupling the production of ADP to the oxidation of NADPH via the pyruvate kinase and lactate dehydrogenase reaction (coupled assay), as described (Zheng & Ramirez, 1999b). The reaction mixture (0.7 ml final volume) contained (in mM): Tris 100 (pH 8.0), Mg-ATP 4,  $\text{MgCl}_2$  2, KCl 50, EDTA 0.2, NADH 0.23, phosphoenol pyruvate 1, 1.4 unit pyruvate kinase, 1.4 unit lactate dehydrogenase and about 25–50  $\mu\text{g}$  proteins, and was assayed at 30–31°C. The activity of the  $\text{Na}^+/\text{K}^+$ -ATPase from porcine cerebral cortex was measured similarly by the coupled assay as described above except that 100 mM NaCl was included. In a few cases, the F0F1-ATPase activity was also measured directly from the release of inorganic phosphate ( $\text{P}_i$ ) from ATP as described (Harris, 1987). In this case the reaction solution (0.7 ml) contained (in mM): Tris 100, (pH 8.0), Mg-ATP 4,  $\text{MgCl}_2$  2, KCl 50, EDTA 0.2 and 25–50  $\mu\text{g}$  proteins. The specific F0F1-ATPase activity in all cases was determined in the presence of the enzyme inhibitors, oligomycin or efrapeptin (Linnett & Beechey, 1979). Ouabain was used to determine the specific  $\text{Na}^+/\text{K}^+$ -ATPase activity.

To study possible effects of phytochemicals on the other enzymes used in the coupled assay of ATPase, i.e. pyruvate kinase and lactate dehydrogenase, ATP was omitted from the buffer and the reaction was started by adding 0.2 mM ADP.

#### Assay for ATP synthesis

ATP synthesis was measured by monitoring the increase in absorbance at 340 nm using an  $\text{NADP}^+$ -linked, ADP-regenerating system (Cross & Kohlbrenner, 1978). The reaction mixture (0.7 ml final volume) contained (mM): HEPES 10 (pH 8.0), succinate 20, glucose 20,  $\text{MgCl}_2$  3, AMP 11,  $\text{NADP}^+$  0.75, ADP 1,  $\text{P}_i$  10, 4  $\mu\text{l } \text{ml}^{-1}$  hexokinase and 2  $\mu\text{l } \text{ml}^{-1}$  glucose-6-phosphate dehydrogenase. The reaction was assayed at 30–31°C and started by adding 20  $\mu\text{l}$  of pre-incubated (5 min at 26°C) submitochondrial particles (about 50–61  $\mu\text{g}$  protein) in 20 mM succinate. A high AMP concentration (11 mM) was used in this assay to inhibit the adenylyl kinase activity present in the submitochondrial particle preparations, which might interfere with measurement of respiration-dependent ATP synthesis by the ATP synthase. The preparations had ATP synthase activity of 0.057–0.083  $\mu\text{mol min}^{-1}$  (mg protein) $^{-1}$ .

To exclude the possible effects of resveratrol on hexokinase and glucose-6-phosphate dehydrogenase used in the assay, the response induced by 140  $\mu\text{M}$  ATP in the absence of submitochondrial particles were tested; these were not significantly affected.

#### Data analysis

To obtain reaction rates, the steady-state linear range of the absorbance change with respect to time was used for both ATPase and ATP synthase activity. A molar extinction coefficient of  $6.22 \times 10^3 \text{ M cm}^{-1}$  was used for  $\text{NAD(P)H}$  to calculate the activity in  $\mu\text{mol ATP hydrolysed or synthesized per min per mg protein}$ . The responses were very stable as judged from control trials performed before, during and after the experiments (less than 4% variations). Data from three or

more experiments were expressed as means (standard deviation (s.d.)). Statistical analysis was performed using Student's *t*-test to compare two groups and analysis of variance (ANOVA) with *post-hoc* Tukey test for comparison of three or more groups.  $P < 0.05$  was considered significant. For concentration-dependent effects of resveratrol with an experimental number of 3,  $IC_{50}$  values are quoted as geometric mean with 95% confidence interval (c.i., based on *t* distribution).

## Results

### Inhibition of rat F0F1-ATPase/ATP synthase by resveratrol

Mitochondrial fractions prepared from both female rat brains and livers were solubilized with 1% digitonin or 1% CHAPS to obtain functional solubilized mitochondrial fractions containing high F0F1-ATPase activity with little  $Na^+/K^+$ -ATPase activity in a coupled ATPase activity assay (Zheng & Ramirez, 1999b). Resveratrol, a phytoalexin present in high concentrations in red wine (usually 10–50  $\mu M$ ) and grapes, rapidly (within 1–2 min) inhibited the brain mitochondrial F0F1-ATPase activity in a concentration-dependent fashion in the range 0.7–70  $\mu M$ , with  $IC_{50}$  values of 18.5  $\mu M$  (geometric mean, 95% c.i. 16.7–20.6  $\mu M$ ;  $n = 3$ ) and 13.0  $\mu M$  (8.80–19.3  $\mu M$ ;  $n = 3$ ) for digitonin-solubilized and CHAPS-solubilized preparations, respectively (Figure 2A,B). With 0.7  $\mu M$  of resveratrol, the inhibition was already significant (5–7% inhibition,  $P < 0.01$ ). Similar results were obtained with both digitonin- and CHAPS-solubilized liver mitochondrial preparations ( $IC_{50}$  of 12 and 21  $\mu M$ , respectively). The inhibitory effect of resveratrol appears to be specific to F0F1-ATPase given that this effect is blocked by oligomycin, an F0F1-ATPase/ATP synthase inhibitor (Linnett & Beechey, 1979). In the presence of 7  $\mu g ml^{-1}$  oligomycin alone, the residual ATP hydrolysis activity in the digitonin-solubilized preparation was 16.1 (0.3%) (mean(s.d.);  $n = 3$ ) when the control activity was assumed to be 100%. In the presence of both 7  $\mu g ml^{-1}$  oligomycin and 21  $\mu M$  resveratrol, the residual activity was 15.8 (0.4%) ( $n = 3$ ). Efrapeptin, a specific inhibitor of the F0F1-ATPase/ATP synthase (Linnett & Beechey, 1979), completely inhibited the enzyme activity at 1–2  $\mu M$  (Figure 2B). Therefore, the effect of resveratrol must be on the oligomycin and efrapeptin-sensitive ATPase (i.e. F0F1-ATPase) activity of this preparation, which corresponded to an average of 83.5 (2.2%) ( $n = 7$ ) of the total ATP hydrolysis activity. Resveratrol itself did not have any effect on the activity of the other enzymes, i.e. pyruvate kinase and lactate dehydrogenase, used in the ATPase activity assay because, in the absence of mitochondrial preparations, 21  $\mu M$  resveratrol did not affect the ADP-induced NADH oxidation. In the presence of 21  $\mu M$  resveratrol, the ADP-induced response was 100.2 (1.0%) ( $n = 3$ ) of control.

To examine the effects of resveratrol on ATP synthesis by the F0F1-ATPase/ATP synthase we used a brain submitochondrial fraction. In the presence of 11 mM AMP the brain submitochondrial particles contained little adenylate kinase activity: more than 95% of the activity corresponded to ATP synthase as shown in the presence of 2  $\mu M$  efrapeptin (data not shown). Resveratrol inhibited ATP synthesis with an  $IC_{50}$  of 27.7  $\mu M$  (22.0–34.8  $\mu M$ ; Figure 2C) and had no effect on the other enzymes used in the assay. Resveratrol also inhibited the F0F1-ATPase activity of the submitochondrial particles with an  $IC_{50}$  of 21.6  $\mu M$  (20.6–22.8  $\mu M$ ; Figure 2C). Therefore, it seems that resveratrol inhibited both ATP synthesis and

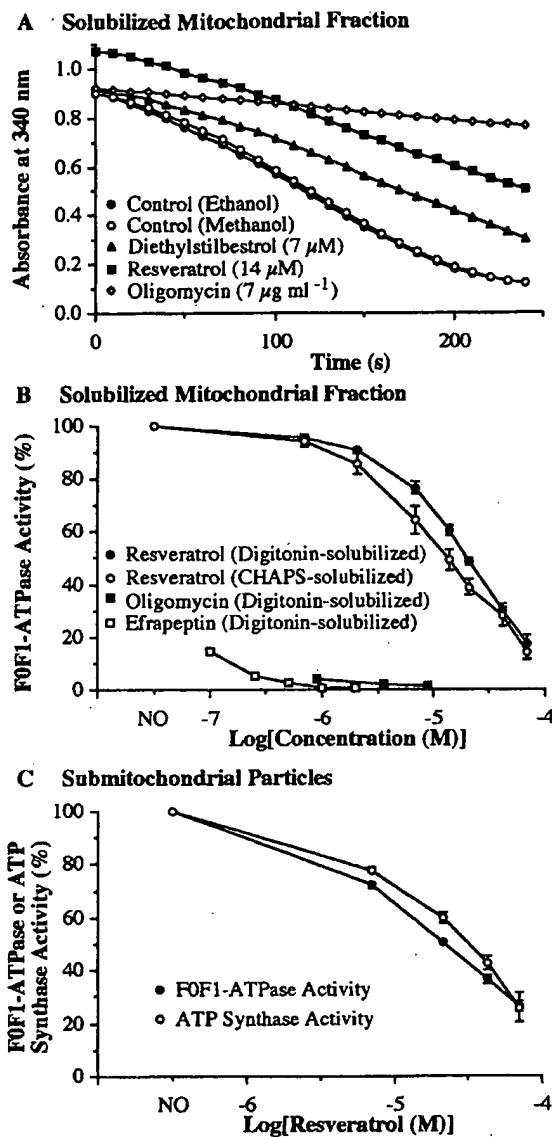


Figure 2 Effects of resveratrol on F0F1-ATPase/ATP synthase activity of rat brain mitochondrial preparations. (A) Typical examples of spectrophotometric read-out showing the absorbance change at 340 nm induced by 20  $\mu M$  digitonin-solubilized brain mitochondrial preparation (51  $\mu g$  protein) in the presence of ethanol vehicle, methanol vehicle, 7  $\mu g ml^{-1}$  oligomycin in methanol, 7  $\mu M$  diethylstilbestrol in ethanol and 14  $\mu M$  resveratrol in ethanol. Resveratrol itself and, to a lesser degree, diethylstilbestrol and oligomycin, had extinction at 340 nm, so the starting point had higher absorbance. Diethylstilbestrol was included here as a comparison. Oligomycin and efrapeptin (see below and text) were included for determination of the F0F1-ATPase activity in the preparations. (B) The concentration-dependent effect of 0.7–70  $\mu M$  resveratrol on digitonin- and CHAPS-solubilized rat brain mitochondrial preparations. Data are expressed as means (s.d.) from three experiments. Also shown is the concentration-dependent effect of oligomycin and efrapeptin. For studying the effect of efrapeptin on ATPase activity, efrapeptin was pre-incubated at 26°C with the solubilized preparation for 6 min to allow for binding. The F0F1-ATPase activity in 0.7% vehicle (this and following figures) was defined as 100%. (C) Effect of resveratrol on ATP synthesis and hydrolysis catalysed by rat brain submitochondrial preparations. F0F1-ATPase activity of rat brain submitochondrial preparations was determined similarly as solubilized mitochondrial preparation except that 2  $\mu g ml^{-1}$  antimycin A was present. Data are expressed as means (s.d.) from three experiments. The F0F1-ATPase/ATP synthase activity in vehicle was defined as 100%.

hydrolysis by the enzyme, but the effect on the ATP synthase activity is slightly less than on the ATPase activity.

Direct measurement of  $P_i$  release from ATP catalysed by digitonin-solubilized brain mitochondrial preparation indicated that 70  $\mu M$  resveratrol inhibited the oligomycin and efrapeptin-sensitive F0F1-ATPase activity by 84.6%, a value similar to that determined by the coupled assay.

#### Differential inhibition of F0F1-ATPase by polyphenolic phytochemicals

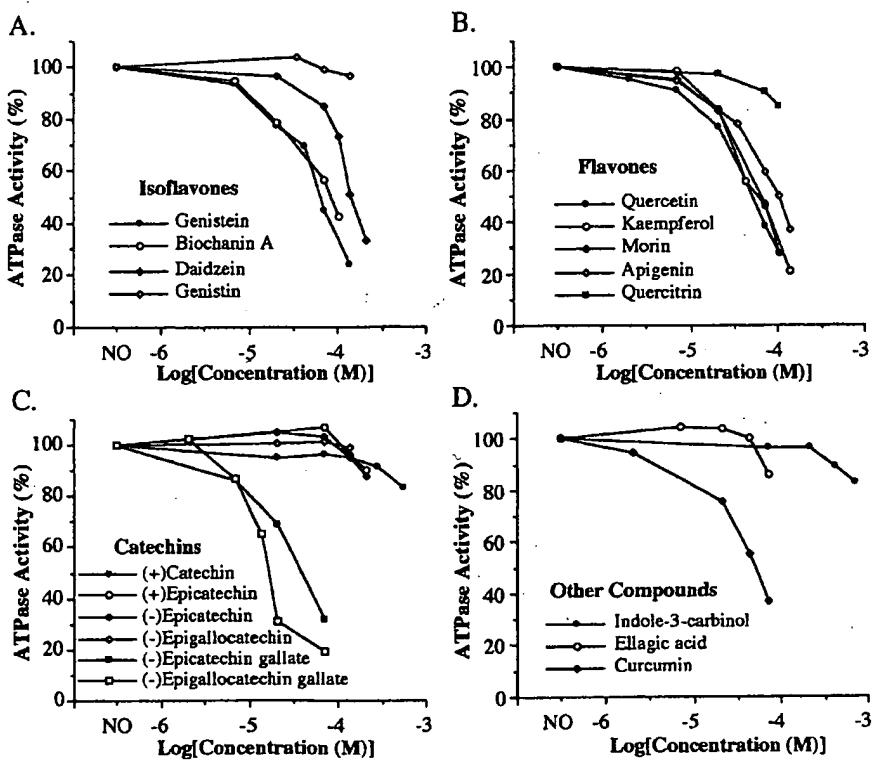
We further screened other phenolic phytochemicals for their effects on the activity of the ATPase in digitonin-solubilized rat brain mitochondrial preparation (Figure 3, Table 1). Three isoflavones, genistein, biochanin A and daidzein—phytoalexins with protein anti-tumour effects— inhibited the ATPase activity with  $IC_{50}$  values of 55–127  $\mu M$  (Figure 3A, Table 1). However, the 7-glucose derivative of genistein (genistin) was essentially without effect at concentrations up to 140  $\mu M$ . The isoflavone compounds had little effect on ADP-induced responses in the absence of the mitochondrial preparations (<10% effect at 70  $\mu M$ ) and, therefore, their actions are due to effects on the ATPase. Genistein and biochanin A, like resveratrol, did not significantly affect the oligomycin-insensitive ATP hydrolysis activity. In the presence of 7  $\mu g ml^{-1}$  oligomycin, 15.3% residual ATP hydrolysis activity remained. When 70  $\mu M$  genistein or biochanin A was added in the presence of 7  $\mu g ml^{-1}$  oligomycin, the residual ATP hydrolysis activity

was 13.4 and 12.9%, respectively. We also tested the effects of genistein on ATP synthase activity and found that at 70  $\mu M$  it also inhibited the enzymatic activity by 62%. Therefore, like resveratrol, genistein inhibits both ATPase and ATP synthase activity of the enzyme.

Consistent with earlier findings (Lang & Racker, 1974; Di Pietro *et al.*, 1975), aglycone flavones also inhibited the brain F0F1-ATPase activity with  $IC_{50}$  values of 50–105  $\mu M$ , while quercitrin had little effect (Figure 3B). When several catechins were tested it was found that diphenoxy catechin, epicatechin and epigallocatechin had little effect on the F0F1-ATPase activity, while the gallate esters, including (–)epigallocatechin gallate (EGCG) and (–)epicatechin gallate (ECG) are potent inhibitors (Figure 3C). Other active inhibitors of F0F1-ATPase include curcumin (Figure 3D), phloretin, tea theaflavins, tannic acid and GSPE (Table 1). Ellagic acid had a modest inhibitory effect on F0F1-ATPase activity (14% at 70  $\mu M$ ) (Figure 3D). Monophenolic compounds, such as caffeic acid, gallic acid and salicylic acid, and nonphenolic compounds, such as indole-3-carbinol (I3C) and tartaric acid, had little effect at similar concentrations (Table 1).

#### Kinetic mechanism of inhibition by resveratrol and genistein

We further examined the effects of resveratrol and genistein on the ATP-dependence of the inhibition of F0F1-ATPase. As shown in Figure 4, the control Lineweaver-Burk plot revealed



**Figure 3** Concentration-dependent effects of selected phytochemicals on total ATPase activity of digitonin-solubilized rat brain mitochondrial preparations. These compounds include isoflavones (A), flavones (B), catechins (C) and several others (D). Experiments were conducted as in Figure 2. Control experiments indicated that (–)epicatechin-gallate, tannic acid and several flavonoids affected ADP-induced responses, i.e. pyruvate kinase or lactate dehydrogenase; therefore, the total ATPase activity was used here. The inhibition of ADP-induced responses was from 28 to 76% at 70  $\mu M$  with kaempferol < apigenin < quercetin < (–)epicatechin gallate < morin. This inhibition, however, will not significantly affect the estimation of  $IC_{50}$  for the ATPase since ATP hydrolysis was the limiting step and the residual oligomycin-insensitive ATPase activity is low (13.4 (1.1)% of total activity). 'NO' indicates the absence of phytochemicals.

Table 1 Effect of phytochemicals on the mitochondrial F0F1-ATPase activity

Compound	$IC_{50}$ ( $\mu\text{M}$ )	Compound	$IC_{50}$ ( $\mu\text{M}$ )
Stilbenes		Chalcones	
Piceatannol	8	Phloretin	40% at 70 $\mu\text{M}$
Resveratrol	19	Phloridzin	<15% up to 70 $\mu\text{M}$
Isoflavones		Other polyphenolics	
Genistein	55	Tannic acid	5 $\mu\text{g ml}^{-1}$
Biochanin A	65	Theaflavins	20 $\mu\text{g ml}^{-1}$
Daidzein	127	GSPE	30 $\mu\text{g ml}^{-1}$
Genistin	<5% up to 140 $\mu\text{M}$	Curcumin	40
Flavones		Ellagic acid	<15% up to 70 $\mu\text{M}$
Quercetin	50	Monophenolics	
Kaempferol	55	Caffeic acid	<15% up to 280 $\mu\text{M}$
Morin	60	Gallic acid	<15% up to 280 $\mu\text{M}$
Apigenin	105	Salicyclic acid	<5% up to 700 $\mu\text{M}$
Quercitrin	<15% up to 105 $\mu\text{M}$	Non-phenolics	
Catechins		Indole-3-carbinol	<15% up to 420 $\mu\text{M}$
EGCG	17	Tartaric acid	<5% up to 7000 $\mu\text{M}$
ECG	45		
(-)Epigallocatechin	<5% up to 140 $\mu\text{M}$		
(-)Epicatechin	<15% up to 210 $\mu\text{M}$		
(+)Epicatechin	<15% up to 210 $\mu\text{M}$		
(+)Catechin	<15% up to 280 $\mu\text{M}$		

The F0F1-ATPase activity of digitonin-solubilized rat brain mitochondrial preparations was determined using an NADH-linked ATP regeneration system. Control experiments were performed in the presence of same amount of vehicle and the variation between control trials was less than 4%. The effect of piceatannol (Zheng & Ramirez, 1999c) and several flavones (Lang & Racker, 1974; Di Pietro *et al.*, 1975) have been shown previously.

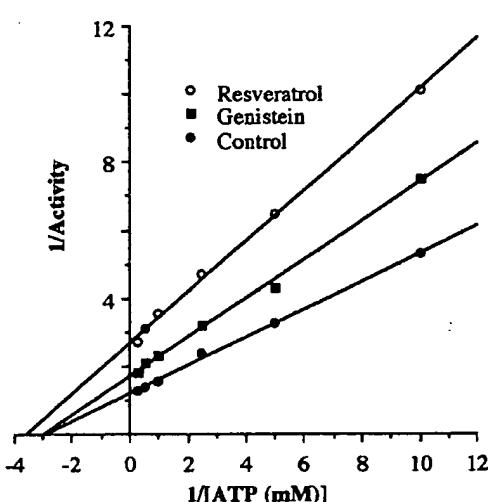


Figure 4 Inhibition of F0F1-ATPase of digitonin-solubilized brain mitochondrial preparation by resveratrol (7  $\mu\text{M}$ ) and genistein (50  $\mu\text{M}$ ) at several concentrations of 0.1–4 mM ATP as shown by Lineweaver–Burk plots. The linear correlation coefficients for all three groups are between 0.996 and 0.999. Note the different  $V_{\max}$  among the control, resveratrol and genistein, and the change in  $K_m$  induced by resveratrol. The control F0F1-ATPase activity of the preparation at 4 mM ATP was 0.797 (0.007)  $\mu\text{mol ATP hydrolysed min}^{-1}$  ( $\text{mg protein})^{-1}$  from three trials performed before, during and after the experimental trials.

maximal specific activity ( $V_{\max}$ ) for this preparation of 0.834  $\mu\text{mol ATP hydrolysed min}^{-1}$  ( $\text{mg protein})^{-1}$  with Michaelis constant ( $K_m$ ) of 0.344 mM. In the presence of resveratrol (7  $\mu\text{M}$ ),  $V_{\max}$  was drastically reduced to 0.370  $\mu\text{mol ATP hydrolysed min}^{-1}$  ( $\text{mg protein})^{-1}$ .  $K_m$  was also reduced by 20% (0.277 mM) in the presence of 7  $\mu\text{M}$  resveratrol, suggesting that resveratrol results in mixed inhibition of F0F1-

ATPase. Genistein (50  $\mu\text{M}$ ) also reduced the  $V_{\max}$  to 0.580  $\mu\text{mol min}^{-1}$  ( $\text{mg protein})^{-1}$ , while  $K_m$  was relatively unchanged (0.331 mM; 3.8% decrease), suggesting that genistein behaves like a classical non-competitive inhibitor.

#### Additive effect of polyphenolic compounds

To determine if resveratrol and other phytochemicals additively contribute to the inhibitory effect of dietary phytochemicals on F0F1-ATPase, we examined the effect of a combination of resveratrol with other active inhibitors at suboptimal concentrations. A combination of suboptimal concentrations of resveratrol, quercetin and kaempferol, which are present in red wine, had an additive inhibitory effect resulting in 56% inhibition as compared to single compounds alone (23, 19 and 17% inhibition for 7  $\mu\text{M}$  resveratrol, 21  $\mu\text{M}$  quercetin and 21  $\mu\text{M}$  kaempferol, respectively) ( $P < 0.001$ ). Similar additive effects were found for resveratrol and genistein, two compounds that are usually not present in the same diet; the combination of 11  $\mu\text{M}$  resveratrol and 35  $\mu\text{M}$  genistein resulted in 58% inhibition as compared to 36% with resveratrol and 26% with genistein alone ( $P < 0.001$ ). Therefore, the presence of these or other active compounds in diet could potentially act together to alter the enzymatic activity of the F0F1-ATPase *in vivo* with considerably lower concentrations of individual compounds.

#### Lack of effect of resveratrol and genistein on $\text{Na}^+/\text{K}^+$ -ATPase

To determine if resveratrol and genistein inhibit the activity of other ATPases, we examined their effect on  $\text{Na}^+/\text{K}^+$ -ATPase from porcine cerebral cortex. Resveratrol and genistein had little effect on this enzyme's activity (Figure 5). Therefore, the stilbene resveratrol and the isoflavone genistein are rather specific in their actions on mitochondrial ATPase/ATP synthase, unlike flavones such as quercetin, which were shown

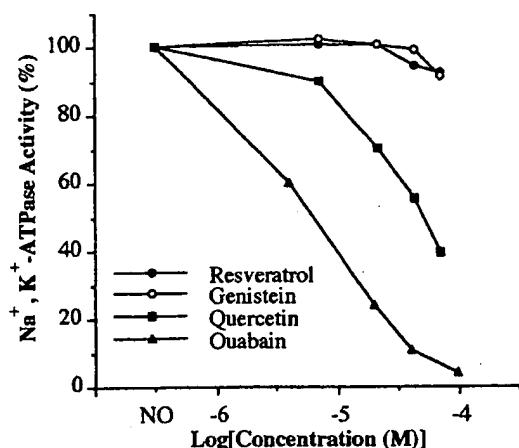


Figure 5 Effects of resveratrol, genistein and quercetin on porcine cerebral cortex  $\text{Na}^+/\text{K}^+$ -ATPase. Each point corresponds to a single trial except for control experiments, which were conducted at least three times with variation less than 3%.

to affect many ATPases, including  $\text{Na}^+/\text{K}^+$ -ATPase (Figure 5) and several other enzymes such as 5'-nucleotidase, phosphor-ylose kinase and protein kinase C, while genistein did not (Akiyama *et al.*, 1987).

## Discussion

By using both rat brain and liver mitochondrial preparations, we have tested several groups of naturally occurring polyphenolic compounds on the activity of rat mitochondrial F0F1-ATPase/ATP synthase. Our studies demonstrate that F0F1-ATPase/ATP synthase is a common target site for resveratrol from red wine, aglycone isoflavones (genistein, biochanin A and daidzein) from soybean, gallate esters of catechins from many sources, and several other polyphenolic compounds at high nanomolar to low micromolar concentrations similar to those used in many biological studies (e.g. Barnes & Peterson, 1995; Jang *et al.*, 1997; Yang *et al.*, 1999). The effect of resveratrol is consistent with recent demonstration of its inhibitory effect on the ATPase activity of intact rat brain mitochondria (Zini *et al.*, 1999). Our recent studies indicate that resveratrol, like piceatannol and quercetin, inhibits the F0F1-ATPase activity by targeting F1, while genistein most likely targets F0 (Zheng & Ramirez, 1999c). Therefore, there are two potential binding sites in ATP synthase for polyphenolic phytochemicals. Interaction of phytochemicals with these binding sites could be a novel mechanism for the actions of these phytochemicals.

Comparison of several groups of polyphenolic phytochemicals indicate the importance of hydroxyl groups in particular positions. For example, three isoflavones – genistein, biochanin A and daidzein – inhibited the ATPase activity with  $\text{IC}_{50}$  values between 55 and 127  $\mu\text{M}$ , while the 7-glucose derivative of genistein (genistin) was essentially without effect up to 140  $\mu\text{M}$ . This, together with the fact that all three active isoflavones contain a 7-OH group, suggests that the 7-OH group is essential for the inhibitory effect of isoflavones. Omission of the 5-OH group in daidzein ( $\text{IC}_{50} = 127 \mu\text{M}$ ) resulted in lower activity in comparison with genistein ( $\text{IC}_{50} = 55 \mu\text{M}$ ). However, modification of the 4'-OH group to 4'-OCH<sub>3</sub> in biochanin A ( $\text{IC}_{50} = 65 \mu\text{M}$ ) seems to have relatively little effect. It seems that a *meta*-quinonoid structure could be essential for the

inhibitory activity. Similarly to genistin, the replacement of quercetin's R1-OH group by *O*-rhamnoside in quercitrin also resulted in much lower inhibitory activity. On the other hand, addition of a phenolic group in catechins converted inactive catechins to active catechins (the gallate esters, EGCG and ECG). The lack of inhibitory activity of monophenolic compounds tested suggests that the inhibition of F0F1-ATPase by phenolic phytochemicals requires two or more phenolic structures.

The levels of several phytochemicals in human body fluids have been determined. For instance, both genistein and daidzein (both free and conjugated forms) reached up to 7  $\mu\text{M}$  in human plasma after soy meals (Xu *et al.*, 1995; Watanabe *et al.*, 1998; King & Bursill, 1998). Administration of decaffeinated green tea could result in maximal plasma concentrations of 0.7 and 1.8  $\mu\text{M}$  in human volunteers for EGCG and EGC, respectively (Yang *et al.*, 1999). However, the levels of these compounds in saliva could reach 10–48 and 38–143  $\mu\text{M}$ , respectively (Yang *et al.*, 1999). These data indicate that much higher concentrations of phytochemicals are present in the oral cavity, and likely in the oesophagus, and that they could be important for the application of tea in the prevention of oral and oesophageal cancers (Yang *et al.*, 1999). Many of these polyphenolic phytochemicals are present in the same plant or food source or may be taken together. For example, red wine contains resveratrol, quercetin, kaempferol and catechins. Therefore, it is important to know that the effects of these compounds on ATPase are additive. This may explain why several brands of red wine and red grape juice, but not white wine, beer or ethanol, inhibit the ATPase activity with  $\text{IC}_{50}$  values of 0.7–1.4%  $\text{v v}^{-1}$  (unpublished data). The lack of effect of white wine on F0F1-ATPase activity is consistent with several earlier studies showing that red wine and grape juice, but not white wine, have cardioprotective and tumour-inhibiting actions in animal models (Demrow *et al.*, 1995) and humans (Furhman *et al.*, 1995; Serafini *et al.*, 1998). Total polyphenolics, including resveratrol and proanthocyanidins, are several-fold lower in white wine than in red wine (Goldberg *et al.*, 1995; Watkins, 1997) which may explain different effects of red and white wine.

Many of these polyphenolic phytochemicals are weak oestrogens (phytoestrogens). For example, resveratrol is a weaker oestrogen receptor  $\alpha$  form agonist, having an effect only when its concentrations are above 3  $\mu\text{M}$  (3–30  $\mu\text{M}$  with  $\text{IC}_{50} = 10 \mu\text{M}$ ) (Gehm *et al.*, 1997). The aglycone isoflavones are more potent phytoestrogens than resveratrol, with their affinity for oestrogen receptor  $\alpha$  and  $\beta$  forms in the following rank order: genistein > daidzein > biochanin A (Kuiper *et al.*, 1998). While biochanin A had a similar inhibitory effect as genistein on F0F1-ATPase activity (Table 1) and tumour growth (Barnes & Peterson, 1995), it has 400 to 8700 fold lower affinity than genistein for oestrogen receptor  $\alpha$  and  $\beta$  forms (Kuiper *et al.*, 1998). Therefore, it is likely that oestrogen receptors are not involved in anti-growth actions of these phytochemicals on cultured cancer cells and that the structure-activity relationship for F0F1-ATPase/ATP synthase inhibition by these phytochemicals is different from that for affinity at oestrogen receptors.

Besides its oestrogen receptor-mediated actions, genistein also inhibits protein tyrosine kinases, while biochanin A and daidzein are at least 40 times less active than genistein (Akiyama *et al.*, 1987). However, several earlier studies indicated that the tyrosine kinase was not required for the inhibitory effect of genistein on tumour growth (Barnes & Peterson, 1995; Stevens *et al.*, 1994; Shao *et al.*, 1998), nor for biochanin A and daidzein. Other mechanisms have been

suggested, including the inhibition of cyclo-oxygenase-1 (Jang *et al.*, 1997) and cytochrome P450 1A1 (Ciolino & Yeh, 1999; Chun *et al.*, 1999) by resveratrol, and inhibition of 5 $\alpha$ -reductase by genistein and biochanin A (Evans *et al.*, 1995). However, the involvement of these and other mechanisms in both cardioprotection and tumour inhibition by diphenolic phytochemicals is unclear.

Several flavones have been shown to interact with other ATPases, such as  $\text{Ca}^{2+}$ -ATPase (McKenna *et al.*, 1996) and  $\text{Na}^+/\text{K}^+$ -ATPase (Hirano *et al.*, 1989), in addition to their effects on F0F1-ATPase (Lang & Racker, 1974; Di Pietro *et al.*, 1975). However, resveratrol and genistein had little effect on  $\text{Na}^+/\text{K}^+$ -ATPase, as shown in Figure 5. On the other hand, ellagic acid has been shown to increase the activity of  $\text{Ca}^{2+}$ -ATPase and  $\text{Ca}^{2+}$  uptake in cardiac sarcoplasmic reticulum ( $\text{EC}_{50}=10 \mu\text{M}$ ) (Antipenko *et al.*, 1999). However, ellagic acid has little effect on F0F1-ATPase of rat brain solubilized mitochondrial preparation at similar concentrations.

Our findings indicate that inhibition of mitochondrial F0F1-ATPase/ATP synthase could be a potential mechanism contributing to the many effects of dietary polyphenolics. It has been shown that the same ATP synthase is localized also on the plasma membranes of several tumour cell lines (Das *et al.*, 1994) and human umbilical vein endothelial cells, and that it is a binding protein for angiostatin, a potent protein inhibitor of angiogenesis and cell proliferation (Moser *et al.*, 1999). Therefore, phytoestrogens could also play a similar role.

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by binding the ATP synthase on the plasma membrane. In fact, genistein has been shown to be a potent inhibitor of angiogenesis (Fotsis *et al.*, 1993). Moreover, resveratrol and genistein also inhibit the bovine mitochondrial NADH:ubiquinone oxidoreductase and induce ornithine decarboxylase, a marker for cancer chemopreventive potency, with  $\text{IC}_{50}=30 \mu\text{M}$  (Fang & Casida, 1998). The simultaneous effects of these phytochemicals on NADH:ubiquinone oxidoreductase and F0F1-ATPase/ATP synthase could significantly affect mitochondrial function and alter ATP level, mitochondrial transmembrane potential and generation of reactive oxygen species, which have been implicated in many cellular processes such as cellular protection, apoptosis,  $\text{O}_2$  sensing and ageing (Wallace, 1999). Further examinations of the effects of the phytochemicals on mitochondrial functions will, therefore, be necessary to identify precisely the mechanisms involved.

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### Gastric H<sup>+</sup>, K<sup>(+)</sup>-ATPase inhibition by catechins.

**Murakami S, Muramatsu M, Otomo S.**

Research Center, Taisho Pharmaceutical Co. Ltd, Ohmiya, Japan.

Five catechins, (+)-catechin, (-)-epicatechin, (-)-epicatechin gallate, (-)-epigallocatechin and (-)-epigallocatechin gallate, inhibited gastric H<sup>+</sup>, K<sup>(+)</sup>-ATPase activity with IC<sub>50</sub> values ranging from 1.7 x 10<sup>(-4)</sup> to 6.9 x 10<sup>(-8)</sup> M, with (-)-epigallocatechin gallate as the most potent inhibitor. The intensity of inhibitor activity paralleled the number of phenolic hydroxy groups in the molecule. The inhibition of the enzyme by (-)-epicatechin was competitive with respect to ATP and noncompetitive with respect to K<sup>+</sup>. These findings suggest that the anti-secretory and anti-ulcerogenic effects of catechins previously reported, are due to their inhibitory activity on gastric H<sup>+</sup>, K<sup>(+)</sup>-ATPase.

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